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(57) Abstract

Described are proteins having molecular weights of 85 and 160 kDa, which proteins are responsive to cold acclimation or drought stress in plants. The cDNA for the 85 and 160 kDa proteins, designated *CAP85* and *CAP160*, are disclosed. Transgenic cells, including microorganisms and plants, can be produced which express the *CAP85* and *CAP160* proteins and thereby advantageously enhance the cold or water stress tolerance in the transgenic organism. Freeze and desiccation damage can also be prevented by applying a cold acclimation protein to the organism needing such protection.

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DESCRIPTION

NOVEL GENES, POLYPEPTIDES, AND COMPOSITIONS FOR COLD TOLERANCE AND DROUGHT RESISTANCE IN PLANTS

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Background of the Invention

Plants must possess and maintain adaptive mechanisms to ensure survival during periods of adverse environmental conditions. Two common stresses that temperate species are most likely to encounter are freezing and drought, both of which may cause cellular dehydration. Plants with the ability to become more freezing tolerant upon exposure to low nonfreezing temperatures in the range of 0-10RC contain genes that encode products which are directly or indirectly responsible for the greater resistance. Low temperature exposure and/or a change in day length may be necessary for these genes to become activated and produce gene product(s) in amounts that are physiologically significant in response to a natural environment. Once activated, proper expression of the genes confers on plant cells and tissues added resistance to the stresses and mechanical strains caused by the withdrawal of cellular water during ice formation. As long as expression of these genes continues at proper levels, and their respective products are localized at the proper cellular sites, the cells will remain tolerant to extracellular freezing at a level that is characteristic for that particular species. Once expression of the genes is reduced or halted in tolerant plants, resistance to the stresses and strains of extracellular freezing will decline. Since this increased freezing tolerance results from the process termed cold acclimation, genes responsible for the greater freezing tolerance that are induced or activated by low temperature are given the name "cold acclimation genes."

Cold acclimation constitutes an inducible response on the part of temperate plants to developing unfavorable temperature conditions. Conversely, most plants that have evolved in and are native to tropical regions of the world lack the ability to tolerate even the slightest freezing. More importantly, they lack the inducible mechanism of cold acclimation and cannot alter their freezing tolerance upon exposure to low nonfreezing temperatures. The dichotomies between temperate and tropical species in tolerance to freezing and ability to cold

acclimate are the result of evolutionary pressures and natural selection as plants colonized colder regions of the world. Nevertheless, it is the activation and expression of certain genes in temperate species that is not only necessary, but is also diagnostic of greater freezing tolerance.

Specific polypeptides are induced and/or synthesized at higher rates only when certain plants and certain tissues are becoming more cryotolerant (Guy, C.L., D. Haskell [1987] *Plant Physiol.* 84:872-878; Guy *et al.* [1988] *Cryobiology* 25:264-271; Gilmour *et al.* [1988] *Plant Physiol.* 87:745-750). Similarly, these same proteins cease to be synthesized at high rates during the loss of cryotolerance at noninductive temperatures. Thus, the synthesis of these proteins shows induction and repression kinetics that exactly mimic the induction and loss of freezing tolerance (Guy and Haskell [1987], *supra*).

Since increases in freezing tolerance are tightly linked to the syntheses of these proteins, the ability of a given plant or tissue to show increases in freezing tolerance ultimately depends on the presence and proper expression of the genes that encode the above proteins inducible by low nonfreezing temperature exposure.

A major component of freezing tolerance appears to involve tolerance to dehydration (Yelenosky, G., C.L. Guy [1989] *Plant Physiol.* 89:444-451). Therefore, responses evoked by water stress may also be involved in freezing tolerance mechanisms. Many studies have demonstrated that a mild drought stress can increase freezing tolerance (Chen, P., P.H. Li, C.J. Weiser [1975] *Hort. Sci.* 10:372-374; Cloutier, Y., C.J. Andrews [1984] *Plant Physiol.* 76:595-598; Cloutier, Y., D. Siminovitch [1982] *Plant Physiol.* 69:256-258; Guy, C., D. Haskell, L. Neven, P. Klein, C. Smelser [1992] *Planta* 188:265-270; Siminovitch, D., Y. Cloutier [1982] *Plant Physiol.* 69:250-255) and that application of abscisic acid (ABA) at non-acclimating temperatures can also increase freezing tolerance (Chen, H.H., P. Gavinlertvatana, P.H. Li [1979] *Bot. Gaz.* 140:142-147; Chen, H.H., L.V. Gusta [1983] *Plant Physiol.* 73:71-75; Lang, V., P. Heino, E.T. Palva [1989] *Theor. Appl. Genetics* 77:729-734; Mohapatra, S.S., L. Wolfraim, R.J. Poole, R.S. Dhindsa [1988] *Plant Physiol.* 89:375-380).

A number of genes responsive to heat stress, water stress, and ABA treatments have now been characterized (U.S. Patent Nos. 5,071,962; 4,797,359; Baker, J., C. Steele, L. Dure III [1988] *Plant Mol. Biol.* 11:277-291; Harada, J.J., A.J. DeLisle, C.S. Baden, M.L. Crouch [1989] *Plant Mol. Biol.* 12:395-401; Hong, B., S.J. Uknes, T.D. Ho [1988] *Plant Mol. Biol.* 11:495-506; Hughes, D.W., G.A. Galau [1991] *Plant Cell* 3:605-618; Mundy, J., K. Yamaguchi-Shinozaka, N.H. Chua [1990] *Proc. Natl. Acad. Sci. USA* 87:1406-1410; Raynal, M., D. Depigny, R. Cooke, M. Delseny [1989] *Plant Physiol.* 91:829-836; Vilardell, J., A.

Goday, M.A. Freire, M. Torrent, M.C. Martinez, J.M. Torne, M. Pages [1990] *Plant Mol. Biol.* 14:423-432). For simplicity of nomenclature, the group of water stress proteins is referred to as WSPs. These proteins were originally identified as LEAs, RABs, and dehydrins. LEAs (late embryogenesis abundant proteins) are, as their name implies, expressed at high levels during the latter stages of seed development and programmed seed dry-down (Dure, L. III, M. Crouch, J. Harada, T.D. Ho, J. Mundy, R. Quatrano, T. Thomas, Z.R. Sung [1989] *Plant Mol. Biol.* 12:475-486). Dehydrins and RABs (responsive to ABA) are similar to LEAs in several ways: hydrophilicity (>55% hydrophilic residues), responsiveness to ABA treatments, boiling solubility, a general absence of cysteine and tryptophan residues, and the presence of repeating motifs. Most of these proteins range in size from 10 kDa to 40 kDa.

In contrast to the water stress proteins, very little is known about proteins and genes which participate in plant freezing tolerance (Cattivelli, L., D. Bartels [1990] *Plant Physiol.* 93:1504-1510; Gilmour, S.J., N.N. Artus, M.F. Thomashow [1992] *Plant Mol. Biol.* 18:13-21; Guy, C.L. [1990] *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 41:187-223; Hajela, R.K., D.P. Horvath, S.J. Gilmour, M.F. Thomashow [1990] *Plant Physiol.* 93:1246-1252; Kurkela, S., M. Franck [1990] *Plant Mol. Biol.* 15:137-144). Recent findings indicate that WSP-like proteins may also participate in plant freezing tolerance (Gilmour, S.J., M.F. Thomashow [1991] *Plant Mol. Biol.* 17:1233-1244; Gilmour *et al.* [1992], *supra*; Nordin, K., P. Heino, E.T. Palva [1991] *Plant Mol. Biol.* 16:1061-1071), but the nature of their role in this process remains uncertain.

Brief Summary of the Invention

The subject invention concerns nucleotide sequences that encode either inducible or upregulated (increased synthesis and accumulation) proteins during exposure to low temperature or under drought stress. Specifically described herein are cDNA sequences encoding the proteins designated *CAP85* and *CAP160*. The subject nucleotide sequences or genes comprising those sequences can be utilized to create transgenic plants having the advantageous characteristics of cold tolerance or drought resistance. These DNA sequences can also be used as probes in assays for crop and plant tolerance levels during seasons of risk to freezing temperature or drought conditions.

Also disclosed are proteins that are encoded by the disclosed genes. These proteins can be employed in novel methods for preventing freeze damage or desiccation damage to a cell, including plant cells and eukaryotic and prokaryotic organisms. Monoclonal antibodies that specifically recognize the disclosed proteins are also described. Further, the subject

invention concerns transgenic plants which have been transformed with the subject genes in order to express the described proteins, thereby enhancing the freezing tolerance or drought resistance of the transformed host.

Brief Description of the Drawings

Figure 1. Peptide sequences of *CAP85*. *CAP85* was cleaved by CNBr and the peptides were resolved by SDS-PAGE. Three major peptides of 66, 50, and 55 kDa were sequenced using gas-phase amino acid sequencing.

Figure 2 shows the sequence of *CAP85* PCR clone pcr733.

Figures 3A and 3B show the sequence of the 1.8 kb cDNA clone of *CAP85*. The synthetic oligomers that were used to amplify the pcr733 fragment from cDNA are shown in the highlighted boxes. The sequence of pcr733 is identical to the 5Q region, 110-850 bp of the cDNA clone. The arrow above the first methionine of the cDNA indicates a translation start site consensus sequence. Repeating motifs are noted as follows: shaded, lysine rich 22-mer; single underline, 16-mer; double underline, 8-mer.

Figure 4 lists the 11 repeats of the 22 amino acid lysine-rich motif. The residue notations are: p, polar; +, positively charged; —, negatively charged; n, nonpolar; g, glycine. Charged residues are highlighted in the consensus sequence.

Figure 5 shows the enhancement of survival rate following a freeze/thaw stress of *Escherichia coli* XL1-Blue cells expressing a fusion protein for spinach *CAP160*. Squares, wild-type cells containing a pBluescript plasmid without an insert; closed circles, cells containing pBluescript expressing *CAP160* fusion protein; diamonds, cells containing a pBluescript without an insert or expressing the *CAP160* fusion protein, cooled to —4RC or —10RC, then warmed to 4RC without freezing. Percent survival was based on unfrozen control cells that were kept at 4RC.

Brief Description of the Sequences

SEQ ID NO. 1 is the nucleotide sequence for *CAP85*.

SEQ ID NO. 2 is the deduced amino acid sequence of *CAP85*.

SEQ ID NO. 3 is the nucleotide sequence for *CAP160*.

SEQ ID NO. 4 is the deduced amino acid sequence of *CAP160*.

SEQ ID NO. 5 is degenerate synthetic olig nucleotide primer 55-A5Q, constructed from the peptide sequence of the CNBr peptides.

SEQ ID NO. 6 is degenerate synthetic oligonucleotide primer 50-B3Q, constructed from the peptide sequence of the CNBr peptides.

Detailed Disclosure of the Invention

The subject invention cocncerns materials and methods for protecting cells against damage that can result from low temperatures or desiccation. Specifically, the invention described herein provides proteins that are useful in procedures for making cells more resistant to cold or drought. Genes encoding these proteins are also provided.

As disclosed herein, the proteins of the subject invention can be purified by preparative electrophoretic methods, and polyclonal and monoclonal antibodies made to the purified proteins. The proteins may also be produced by recombinant menas. Specifically described herein are the *CAP85* and *CAP160* proteins and the genes encoding these proteins.

CAP85. *CAP85* is a basic protein with an apparent molecular weight of about 85 kDa in 10% acrylamide gels and exhibiting a pI of about 6.3 in isoelectric focusing gels. The protein is present in spinach leaf tissue and hypocotyl. The mRNA for *CAP85* does not appear to encode a signal sequence or a precursor protein for transport into organelles.. The gene(s) are encoded in the nucleus, and the protein is synthesized on cytoplasmic ribosomes. The DNA sequence for *CAP85* was determined and is shown herein as SEQ ID NO. 1. The corresponding amino acid sequence is shown in SEQ ID NO. 2. Several partial amino acid sequences derived from cyanogen bromide cleavage fragments have also been determined. Western blot analyses indicate an 85 kDa molecular weight protein is present in nonacclimated leaf tissue at low levels, but is accumulated when plants are grown at 5RC. Like the *CAP160* protein, we have found that this protein is also accumulated in leaf tissue subjected to desiccation.

CAP85 is regulated in response to low temperature and is also responsive to slight changes in water balance. *CAP85* accumulates during exposure to low temperature or water stress. In the experiments described herein, the soil was well-watered and the plants did not show any loss of turgor or decrease in water potential. Therefore, the accumulation of *CAP85* during cold acclimation does not appear to be due to water stress, but represents a true low temperature response.

CAP160. *CAP160* is an acidic protein with an apparent molecular weight of about 155 to about 160 kDa in 10% SDS acrylamide gels and also exhibits a pI of about 4.5 to 4.7 in isoelectric focusing gels. This protein is present in spinach leaf and hypocotyl tissue and can be resolved into as many as five physically similar isoforms varying only slightly in pI

and molecular weight. This protein is phosphorylated. The protein is not accumulated in the nucleus, chloroplast, or mitochondria of the cell. It is encoded by a nuclear gene(s) and is synthesized on cytoplasmic ribosomes. The mRNA does not encode a precursor translation product exhibiting a leader sequence for transport into organelles. The DNA sequence for the gene encoding the *CAP160* protein is shown as SEQ ID NO. 3. The amino acid sequence of the protein is shown as SEQ ID NO. 4. Partial amino acid sequences have been derived from cyanogen bromide cleavage fragments of the protein. This protein, or similar homologues, may be present in a number of plant species including *Citrus*, *Poncirus*, *Petunia*, and *Arabidopsis*. Western blot experiments, using mouse hybridoma cell culture supernatants, demonstrate that this protein is present in nonacclimated spinach leaf and hypocotyl tissue, and is accumulated during exposure to 5RC. This protein is also accumulated in droughted or desiccated leaf and hypocotyl tissue.

The amino acid compositions for each of the described proteins was determined and compared in Table 1. Proteins from cold acclimated hypocotyl tissue were separated by two-dimensional gel electrophoresis and electroblotted onto PVDF. Individual proteins were excised, hydrolyzed, and the liberated amino acids were determined by HPLC.

Table 1. Amino acid composition of high molecular mass cold acclimation proteins from spinach hypocotyl tissue

Amino acid	Composition (mol %)	
	160 kDa	85 kDa
Asparagine/aspartic	12.4	16.2
Threonine	9.8	3.8
Serine	10.6	4.9
Glutamine/glutamic	15.3	16.4
Proline	5.2	5.0
Glycine	14.8	8.5
Alanine	7.0	5.8
Methionine	1.4	0.4
Isoleucine	3.6	2.9
Leucine	4.3	5.8
Histidine	4.6	9.3
Lysine	7.2	12.9
Valine	1.8	3.0
Tyrosine	0.5	3.8
Phenylalanine	0	0.6
Arginine	1.3	0.7

Plasmids containing the nucleotide sequences of the subject invention were deposited with the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, MD 20852 USA.

The subject plasmids have been deposited under conditions that assure that access to the plasmids will be available during the pendency of this patent application to one determined by the Commissioner of Patents and Trademarks to be entitled thereto under 37 CFR 1.14 and 35 U.S.C. 122. The deposits are available as required by foreign patent laws in countries wherein counterparts of the subject application, or its progeny, are filed. However, it should be understood that the availability of a deposit does not constitute a license to practice the subject invention in derogation of patent rights granted by governmental action.

Further, the subject deposits will be stored and made available to the public in accord with the provisions of the Budapest Treaty for the Deposit of Microorganisms, i.e., they will

be stored with all the care necessary to keep them viable and uncontaminated for a period of at least five years after the most recent request for the furnishing of a sample of a deposit, and in any case, for a period of at least thirty (30) years after the date of deposit or for the enforceable life of any patent which may issue disclosing the deposits. The depositor acknowledges the duty to replace a deposit should the depository be unable to furnish a sample when requested, due to the condition of the deposit. All restrictions on the availability to the public of the subject deposits will be irrevocably removed upon the granting of a patent disclosing them.

The cDNA transcript can be obtained from the plasmids in essentially pure form by standard art methods. The essentially pure cDNA thus obtained can be used for subcloning into a different transformation vector.

Introduction of a single or multiple genes into plants and appropriate expression can lead to an increase in cold tolerance or drought resistance in the transformed plant. The described transformations center around introduction of the genes using engineered Ti plasmid vectors in *Agrobacterium tumefaciens*. Model libraries containing but a single cold acclimation gene, and all possible combinations of several cold acclimation genes can be used to transform freezing sensitive plants. Expression of the genes can be controlled by a number of promoters ranging from the 35S promoter of cauliflower mosaic virus to a number of inducible promoters where the expression of the introduced genes can be controlled by the external application of an environmental condition or chemical. Alternatively, the natural upstream promoter regions of the cold acclimation genes can be used to control expression of the cold acclimation genes in transgenic plants upon exposure to low temperatures. However, it may be necessary to introduce the genes for the *trans*-acting factors that recognize the cold acclimation consensus elements of the cold acclimation gene promoters, provided that freezing sensitive plants, in general, lack genes responsive to low temperature.

The creation of transformed plants can utilize any one of several strategies well known in the art for introduction of foreign genes into cold sensitive plants, including electroporation and facilitated DNA uptake (protoplast and liposome fusion) (Fromm *et al.* [1986] *Nature* 319:791), the biolistic gun, and with *Agrobacterium* (Horsch *et al.* [1985] *Science* 227:1229-1231). At the present time, transformation with *Agrobacterium* is the most versatile method available. Inoculations of plant tissues to be transformed by Ti plasmid transfer can be made with *Agrobacterium tumefaciens* strains carrying cointegrates of disarmed Ti plasmid constructs encoding genes for antibiotic resistance and a spinach cold acclimation gene. The bacterial strains and transformation vectors have been described and are well known in the art.

The proteins described herein can be used to protect a cell from freeze damage or desiccation damage brought on by the formation of intracellular or extracellular ice. For example, the protein can be added, with an appropriate carrier, directly to the plant by applying it to the surface or injected into the plant or can be placed in the growth medium of the plant. Appropriate carriers, preservatives, and adjuvants are well known in the art and can be readily adapted for each particular protein or plant.

As is well known in the art, the amino acid sequence of a protein is determined by the nucleotide sequence of the DNA. Because of the redundancy of the genetic code, i.e., more than one coding nucleotide triplet (codon) can be used for most of the amino acids used to make proteins, different nucleotide sequences can code for a particular amino acid. Thus, the genetic code can be depicted as follows:

Phenylalanine (Phe)	TTK	Histidine (His)	CAK
Leucine (Leu)	XTY	Glutamine (Gln)	CAJ
Isoleucine (Ile)	ATM	Asparagine (Asn)	AAK
Methionine (Met)	ATG	Lysine (Lys)	AAJ
Valine (Val)	GTL	Aspartic acid (Asp)	GAK
Serine (Ser)	QRS	Glutamic acid (Glu)	GAJ
Proline (Pro)	CCL	Cysteine (Cys)	TGK
Threonine (Thr)	ACL	Tryptophan (Trp)	TGG
Alanine (Ala)	GCL	Arginine (Arg)	WGZ
Tyrosine (Tyr)	TAK	Glycine (Gly)	GGL
Termination signal	TAJ		
Termination signal	TGA		

Key: Each 3-letter deoxynucleotide triplet corresponds to a trinucleotide of mRNA, having a 5Q-end on the left and a 3Q-end on the right. All DNA sequences given herein are those of the strand whose sequence corresponds to the mRNA sequence, with thymine substituted for uracil. The letters stand for the purine or pyrimidine bases forming the deoxynucleotide sequence.

A = adenine

G = guanine

C = cytosine

T = thymine

X = T or C if Y is A or G

X = C if Y is C or T

Y = A, G, C or T if X is C

Y = A or G if X is T

W = C or A if Z is A or G

W = C if Z is C or T

Z = A, G, C or T if W is C

Z = A or G if W is A

QR = TC if S is A, G, C or T; alternatively

QR = AG if S is T or C

J = A or G

K = T or C

L = A, T, C or G

M = A, C or T

The above shows that the amino acid sequences of *CAP85* or *CAP160* can be prepared by nucleotide sequences other than those disclosed. Functionally equivalent nucleotide sequences encoding the novel amino acid sequences of these proteins and fragments can be prepared by known synthetic procedures. Accordingly, the subject invention includes such functionally equivalent nucleotide sequences.

The one-letter symbol for the amino acids used above is well known in the art. For convenience, the relationship of the three-letter abbreviation and the one-letter symbol for amino acids is as follows:

Ala	A	Leu	L
Arg	R	Lys	K
Asn	N	Met	M
Asp	D	Phe	F
Cys	C	Pro	P
Gln	Q	Ser	S
Glu	E	Thr	T
Gly	G	Trp	W
His	H	Tyr	Y
Ile	I	Val	V

Thus, the scope of the subject invention includes not only the specific nucleotide sequences depicted herein, but also all equivalent nucleotide sequences coding for molecules

with substantially the same biological activity. The term "equivalent" is being used in its ordinary patent usage here as denoting a nucleotide sequence which performs substantially as the nucleotide sequence identified herein to produce molecules with substantially the same biological activity in essentially the same kind of hosts. Within this definition are subfragments which have freezing tolerance and drought resistance biological activity.

It is well within the skill of those in the genetic engineering art to use the nucleotide sequences encoding the described environmental stress resistance activity of the subject invention to produce the disclosed proteins via microbial processes. Fusing the sequences into an expression vector and transforming or transfecting into appropriate hosts are standard procedures used in producing other well-known proteins, e.g., insulin, interferons, human growth hormone, and the like. Similar procedures, or obvious modifications thereof, can be employed to prepare proteins by microbial means or plant or mammalian tissue culture technology in accord with the subject invention.

Further, the scope of the subject invention is intended to cover not only the specific amino acid sequences disclosed, but also similar sequences of proteins or protein fragments having comparable biological activity.

Materials and Methods

Plant material. Spinach seedlings (*Spinacia oleracea* L. cv Bloomsdale) were grown from seed in a controlled environment as previously described (Guy and Haskell [1987], *supra*). Cold acclimation, deacclimation, and water stress treatments were conducted as previously described (*Id.*).

Protein extraction. Protein was extracted from etiolated spinach (*Spinacia oleracea* L. cv Bloomsdale) hypocotyl-cotyledon tissue grown at 5RC for 4 weeks. Frozen tissue was ground in a dry-ice-cooled mortar. Forty grams of tissue was homogenized in a Polytron with 100 ml of 80% v/v distilled phenol buffered with 120 mM Tris-HCl (pH 6.8), 50 mM EDTA, 100 mM KCl, 2% v/v Triton X-100, 5% v/v glycerol, and 2% v/v 2-mercaptoethanol was added to an additional 100 ml of the preceding buffer. The extract was centrifuged at 15,000 *g* for 5 minutes. The aqueous phase was removed and the phenol phase extracted with 50 ml buffer 3 additional times. The final phenol phase was filtered through glass wool to remove cellular debris. Protein was precipitated from the phenol solution with 5 volumes of −20RC acetone containing 1% v/v 2-mercaptoethanol for 2 hours at −20RC and pelleted at 15,000 *g* for 5 minutes to remove insoluble material. The supernatant was stored at −20RC. All protein content determinations were by the dye-binding method.

Protein purification. Total protein extracts were fractionated by preparative, free solution isoelectric focusing (Rotofor™, BioRad). Rotofor solution with 2% v/v ampholytes (0.8% pH 5-7, 0.8% pH 4-6, and 0.4% pH 3-10) (BioRad) was prefocused for 1 hour at 12 W constant power with 10RC coolant. The protein sample was dissolved in Rotofor solution with 2% ampholytes (usually 3.5 to 4.0 ml containing from 31 to 187 mg of protein). The protein/ampholyte solution was loaded into the compartment having a pH near the midpoint of the gradient (to substantially eliminate protein precipitation during focusing). Proteins were focused for 5 hours at 12 W constant power with 10RC coolant. After sample collection, 25 µl of each fraction was loaded directly onto, and separated in, 10% SDS-polyacrylamide gels. Separated proteins were stained with Coomassie blue. The free solution isoelectric focusing purification closely approximated analytical two-dimensional separations for spinach cold acclimation proteins, which facilitated identification of fractions containing *CAP85*. Total protein content was assayed and the percentage of *CAP85* present in the fraction was determined by densitometry of the SDS-polyacrylamide gel separation, which yielded an estimate of the micrograms of *CAP85* present. Protein from fractions containing *CAP85* were precipitated with 5 volumes of −20RC acetone with 1% 2-mercaptoethanol for 2 hours at −20RC followed by centrifugation at 11,000 *g*. The co-precipitating urea was removed by washing with 10 ml of methanol. After centrifugation, the methanol-urea supernatant was discarded and the protein pellet dried under vacuum.

Monoclonal antibody production. Protein pellets from fractions of free solution isoelectric focusing containing *CAP85* were resuspended in phosphate buffered saline. Balb/c mice were injected with approximately 100 µg of *CAP85* (500 µg total protein) with RIBI MPL + TDM adjuvant. The four boosts of antigen in adjuvant were made with equal or greater amounts of antigen. Serums were titered and cell fusion supernatants were screened by Western blot of SDS-PAGE fractionated spinach proteins using a miniblotter (Immunetics). Immune complexes were detected using alkaline phosphatase conjugated to goat anti-mouse IgG. The color development substrate was NBT/BCIP.

Polyclonal antibody production. A 3 ml solution containing approximately 2400 µg total protein and about 500 µg of *CAP85* was fractionated on a SDS-polyacrylamide gel (7.5%, 0.75 mm thick, 16 cm wide). After briefly staining with Coomassie blue and destaining, the *CAP85* band was excised, rinsed in water 2 minutes, and homogenized with PBS in a Ten Broeck tissue grinder. The homogenized gel was transferred with water to a glass tube and lyophilized to reduce the volume of liquid. A Balb/c mouse was injected

initially with 70 μg of *CAP85* in RIBI MPL + TDM adjuvant. This was followed by three boosts of 70, 70, and 100 μg of *CAP85* in adjuvant. The final working titer was 1:10,000.

Protein cleavage, sequencing, and homology. Initial attempts to sequence *CAP85* revealed that the amino terminus was blocked. Amino acid composition suggested that the methionine content was low. Therefore, *CAP85* was cleaved at methionine residues with CNBr to obtain sequencable internal peptides. Protein pellets from enriched *CAP85* fractions resulting from free solution isoelectric focusing were redissolved in SDS sample buffer. The protein was loaded onto a 7.5% SDS-polyacrylamide slab gel (0.75 mm thick and 16 cm wide) that had been pre-run with 0.1 mM Na thioglycolate in the cathode buffer until the front was at least 1 cm into the running gel. The proteins were transferred by semi-dry electroblotter to PVDF membrane. Without allowing the blot to dry, the *CAP85* band was excised and cut into pieces to fit into a 1.5 ml microfuge tube. In the fume hood, 1 ml of 70% formic acid (diluted with distilled water from 90% formic acid) was added to the tube followed by 100 μl of 5 M CNBr in acetonitrile. The closed tube was sealed from parafilm, wrapped in foil, and placed on a rocker table. A nitrogen atmosphere was not necessary for CNBr cleavage in the small tube. The reaction was allowed to proceed for 14 hours, then the tube was opened in the hood to vent gases. Formic acid was removed by lyophilization. The solution was transferred to a 15 ml Corex tube and diluted with 3 ml of distilled water, frozen at −80RC and lyophilized overnight. When dry, another 1 ml of distilled water was added to dissolve the residue, then lyophilized again to dryness. The peptide fragments were dissolved in SDS sample buffer. After protein determination, the fragments were fractionated on 10 and 15% SDS-polyacrylamide gels that had been pre-run as before with 0.1 mM Na thioglycolate in the cathode buffer. The protein fragments, along with molecular weight standards, were transferred to PVDF using a semi-dry electroblotter. The membrane was washed twice with distilled water to remove glycine and stained with 0.2 amido black in distilled water and then destained by repeated washing with distilled, deionized water (ddH_2O). When dry, the membranes were stored at −20RC until the fragments were sequenced. Protein sequencing was performed on an Applied Biosystems gas phase sequenator.

Boiling stability. Proteins were homogenized from 1.0 g of 2 day cold acclimated spinach leaf tissue in 2.0 ml of 50 mM MOPS, pH 7.5, 10 mM $MgCl_2$, 1 mM EDTA buffer in a glass tissue grinder held on ice. The homogenate was centrifuged at 15,000 *g* in a microfuge for 10 minutes. An aliquot of the supernatant was subjected to 100RC heat treatment for 2 or 10 minutes. The boiled sample was centrifuged at 15,000 *g* for 5 minutes to remove insoluble proteins. Equal volumes, 15 μl, of total extract and boiled samples were

electrophoresed on a 8.0% SDS-PAGE. Proteins were then electroblotted onto PVDF membrane and probed with the appropriate antibodies or stained with Coomassie blue.

Cell fractionations and protein extractions. Plants were cold acclimated at least 7 days prior to fractionations. All isolation steps were performed on ice or at 4RC. Final fractionation extracts were boiled for 2 minutes, centrifuged for 10 minutes at 15,000 *g*, transferred to new tubes, and stored at −20RC. Protein content was determined by dye-binding method.

Chloroplasts. Chloroplasts were isolated using a modification of the protocol of Cline (Cline, K. [1975] "Purification of inner and outer chloroplast envelope membranes," In *Modern Methods of Plant Analysis New Series Volume 1: Cell Components*, H.F. Linskens, J.F. Jackson, eds., Springer-Verlag, Berlin-Heidelberg). Plants were kept in the dark for at least 24 hours before isolating chloroplasts to reduce the size of starch grains that interfere with recovery of intact chloroplasts. Spinach leaf tissue, 5 g, was homogenized in 20 ml 1X HSB (homogenization solution with BSA, modified to 0.5 M sorbitol to isotonically stabilize cold acclimated chloroplasts) with a Polytron using three 5-7 second bursts at setting 12. The homogenate was filtered through a cotton plugged syringe and divided between two 15-ml Corex tubes that were centrifuged for 2 minutes at 2,500 *g* in Beckman JA 13.1 rotor. The pellets were resuspended in 0.5 ml of 1X HS and layered on Percoll gradients that were formed by mixing 5 ml of Percoll and 5 ml of 2X HSB in plastic tubes centrifuged at 43,000 *g* for 30 minutes in a JA 20 rotor. Chloroplast suspensions were layered on the preformed gradients and centrifuged for 30 minutes at 1,000 *g* in a JA 13.1 rotor. The band of intact chloroplasts from the gradient was pooled and diluted with 2 volumes of 1X HS (homogenization solution). Chloroplasts were recovered by centrifugation in a JA 13.1 rotor at 2,000 *g* for 7 minutes. The chloroplast pellet was resuspended in 320 µl HS, to which was added 80 µl of proteinase K (Boehringer Mannheim) (1 mg/ml HS). After 30 minutes on ice, the proteinase K was inactivated by bringing the solution to 2 mM PMSF. The suspension was centrifuged at 1,500 *g* for 6 minutes in a JA 13.1 rotor and the resultant chloroplast pellet was washed with buffer. Protein from the chloroplast pellet was extracted following the addition of 30 µl of 8 mM PMSF and 90 µl of 1X SDS buffer.

Nuclei. Nuclei were isolated in Honda medium (HM). Nuclei were further purified using a modification of the Percoll step gradient. See Luthe, D.S., R.S. Quatrano (1980) *Plant Physiol.* 65:305-308. Two aliquots of 7 to 8 g of tissue were placed in a glass Petri dish on ice. HM (with 1 mM DTT substituted for the 2% 2-mercaptoethanol) was added in a ration of 3 to 1 (v/w). The tissue was chopped with a razor blade for 10 minutes. The chopped

material was filtered through a coarse screen followed by a fine screen (mesh size not known). Twice, the tissue residue was returned to the Petri dish, washed with 10 ml of HM, and refiltered. The filtrate was centrifuged at 1,000 *g* for 5 minutes in Beckman JA 13.1 swinging bucket rotor in 30 ml Corex tubes. The pellet was resuspended in 5 ml HM in 15 ml Corex tubes and the centrifugation step repeated. The resulting pellet was resuspended in 2 ml of HM.

The HM suspensions were layered on discontinuous gradients of Percoll containing the following steps: 2 ml 40%, 2 ml 60%, and 4 ml 80% (v/v) Percoll made with gradient buffer (0.25 M sucrose, 25 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$). The gradients were centrifuged at 3,948 *g* in a JA 13.1 swinging bucket rotor for 30 minutes. The interfaces of the 40% and 60% Percoll steps were collected and transferred to 15 ml Corex tubes and mixed with 5 ml HM. The suspensions were centrifuged for 5 minutes at 5,685 *g* in a JA 13.1 rotor. The supernatants were aspirated off and each pellet was resuspended in 50 µl gradient buffer and transferred to microfuge tubes. The Corex tubes were rinsed with 25 µl more of buffer and the rinses added to the microfuge tubes. The Corex tubes were rinsed with 25 µl more of buffer and the rinses added to the microfuge tubes. The presence of nuclei was confirmed by light microscopy. Protein was extracted from the nuclei by adding 50 µl of 2 X SDS buffer, which formed a viscous solution, and vortexed. Protein extracts from several gradient purifications were pooled.

Mitochondria. Spinach was grown in the dark to produce etiolated hypocotyls. Four aliquots of 5 g of hypocotyl tissue were chopped for 10 minutes with razor blades in glass Petri dishes on ice containing 15 ml each of chopping medium (CM) (0.3 M mannitol, 1 mM EDTA, 30 mM MOPS-KOH (pH 7.2), and 0.02% defatted BSA (w/v). The solutions of chopped tissue were individually filtered through a coarse screen followed by a fine screen into 50 ml plastic tubes. The tissue residue was returned to the Petri dish, washed with 10 ml CM, filtered into the plastic tubes, and the process repeated with 5 ml CM. The filtered suspensions were centrifuged at 3,000 *g* for 5 minutes in a Beckman JA rotor. The pellets were resuspended with 1.0 ml CM and layered on discontinuous Percoll gradients in 15 ml Corex tubes containing the following steps: 4 ml 13.5%, 4 ml 21%, and 2 ml 45% (v/v) Percoll made with gradient buffers of Jackson *et al.* (Jackson, C., J.E. Dench, D.O. Hall, A.L. Moore [1979] *Plant Physiol.* 64:150-153). The gradients were centrifuged for 30 minutes at 7,500 *g* in a JA 20 rotor. Fractions from the interface of the 21% and 45% steps from each of the two gradients were mixed with 20 ml of chopping medium minus BSA (CM-BSA) in 30 ml Corex tubes and centrifuged at 11,000 g for 15 minutes. After aspiration of most of

the supernatant, loose pellets were transferred to microfuge tubes and spun in JA 18.1 at 11,000 *g* for 15 minutes. Supernatant was removed with a Pasteur pipet. Eighty microliters of CM-BSA and 20 µl of proteinase K (1 mg/ml CM-BSA) was added to the pellet. After 30 minutes on ice, the suspension was brought to 2 mM PMSF, mixed, and centrifuged in a JA 18.1 rotor at 11,000 *g* for 15 minutes. The supernatant was removed and the pellet was washed with 150 µl CM-BSA followed by centrifugation as above. The supernatant was removed. To the pellet, 60 µl of 8 mM PMSF in CM-BSA and 90 µl 1 X SDS buffer were added with mixing by vortex mixer.

Endoplasmic reticulum. Three grams of leaf tissue were ground with a mortar and pestle with 2 vol (w/v) of grinding buffer (10 mM Tris-HCl, pH 8.5 at 25RC, 7.2% sucrose (w/v), 10 mM KCl, 5 mM $MgCl_2$). The solution was centrifuged at 10,000 *g* for 5 minutes in a JA rotor. The supernatant was layered on a discontinuous sucrose gradient of modified grinding buffer: 0.8 ml 0.5 M, 0.8 ml 1.0 M, 0.8 ml 1.5 M, 0.4 ml 2.0 M sucrose. The gradients were centrifuged at 80,000 *g* in SW 50.1 rotor for 30 minutes. The recovered endoplasmic reticulum fraction from the interface of the 1.0 M and 1.5 M sucrose layers was mixed with 10 ml of grinding buffer minus sucrose (GB—S) and centrifuged in a JA 20 rotor for 1 hours at 37,000 *g*. The pellet was resuspended in 900 µl of GB—S and 100 µl of 1.5 mg/ml GB—S stock proteinase K. After 30 minutes on ice, the suspension was brought to 2 mM PMSF and centrifuged in a JA 18.1 rotor for 1 hour at 37,000 *g*. The pellet was mixed in 100 µl of 1 X SDS buffer.

Soluble protein. After the ultracentrifugation step in the endoplasmic reticulum isolation procedure, the sample layer at the top was recovered and deemed the soluble protein fraction. One volume of this fraction was mixed with one volume of 2 X SDS sample buffer.

Leaf and hypocotyl protein. Protein was extracted separately from cold acclimated leaf and hypocotyl tissue in SDS buffer and processed as above. Since *CA P85* has no known enzymatic activity and can only be detected by antibody reactivity, marker proteins for selected cell fractions were assayed by protein blotting. The chloroplast marker was a polyclonal antibody reactive against the large subunit of RuBisCo purchased from Sigma. For ER, a polyclonal antibody specific for the tobacco ER luminal protein, BiP, was used. The mitochondrial marker was a polyclonal antibody reactive against the α-subunit of the F_1-ATPase of yeast, and the nuclear marker was an antibody reactive against high mobility group proteins.

Equal amounts of protein (3 µg/lane) were fractionated on SDS-PAGE gels and marker proteins detected by antibody binding and color visualization as described previously.

Nucleic acid purification. RNA was extracted from spinach tissues using the phenol/LiCl procedure. Poly (A^+) RNA was purified using oligo dT-cellulose and used in RNA blots and cDNA synthesis. DNA was extracted from spinach leaf tissue following methods known in the art.

RNA and DNA blotting. RNA blots were performed using total or poly (A^+) RNA. DNA blots were performed using 20 µg of spinach total DNA digested with 10X excess restriction endonuclease. The digested DNA was electrophoresed in 0.8% agarose gels in TBE. The gels were then pressure blotted to Hybond-N nylon membrane (Amersham) and fixed with UV light (Stratalinker 1800, Stratagene). Both RNA and DNA blots were prehybridized in 50% formamide, 5 X SSPE, 5 X Denhardts, 0.2% SDS, 10 µg/ml salmon sperm DNA at 42RC for at least 4 hours. DNA used in both blotting procedures was labeled using random primers as previously described (52). Blots (11.5 x 12.5 cm) were hybridized with 2 x 10^7 cpm of labeled probe (approximately 5 x 10^5 cpm/ng), overnight at 42RC, then washed twice in 2 X SSC, 0.2% SDS for 15 minutes at room temperature, followed by 2 washes in 0.5 X SSC, 0.2% SDS for 30 minutes at 68RC, and 2 washes in 0.1 X SSC, 0.2% SDS for 15 to 30 minutes at 68RC. Blots were wrapped wet in plastic, and placed into cassettes and exposed XAR5 X-ray film with one intensifying screen at −80RC.

Library construction and screening. A PCR fragment specific for *CAP85* was generated from cDNA synthesized from 2 day cold acclimated spinach leaf tissue poly (A^+) RNA. Synthetic oligonucleotides matching 50 (50-B3Q) and 55 (55-A5Q) kDa CNBr peptide sequences were used to amplify cDNA. Approximately 1/10 of a cDNA synthesis reaction from an initial 2 µg poly (A^+) RNA was used in the amplification reaction. The amplification of cDNA with the synthetic oligonucleotides was performed following a cycling regime of an initial denaturation of 3 minutes at 94RC, followed by 40 cycles of 1 minute 94RC, 1 minute 50RC, 3 minutes 72RC, and finishing with a final 10 minute extension at 72RC. From PCR, a 650 bp fragment was purified and blunt end cloned into *Eco*RV-digested Bluescript (Stratagene). cDNA synthesis for library construction, using RNA from 2 day cold acclimated leaf tissue, was performed with the Uni-Zap unidirectional lambda phage cloning kit (Stratagene). The library was screened using the PCR generated clone for *CAP85*. DNA sequencing was accomplished by the Taq DyeDeoxy™ Terminator Cycle Sequencing Kit (Applied Biosystems, Inc.) on an automated sequencer (Applied Biosystems, Inc.).

Sequence analysis and homology searches. Analysis of DNA sequences was initially carried out using DNASTAR (DNASTAR, Inc.). Searches of gene data bases (GENEBANK,

EMBL, NBRF) and protein data bases (PIR and Swissprot) were carried out using GCG (Genetics Computer Group), which allows comparisons of DNA and peptide sequences.

Following are examples which illustrate procedures, including the best mode, for practicing the invention. These examples should not be construed as limiting. All percentages are by weight and all solvent mixture proportions are by volume unless otherwise noted.

Example 1 — *CAP85* Protein and Gene Characterization

The influence of acclimation and deacclimation on *CAP85* content in leaves was determined by protein blot analyses. Protein levels were elevated throughout the cold acclimation period and decreased following a return to 25RC. There were significant levels of *CAP85* one day after a return to 25RC. After 7 days of deacclimation, the levels of *CAP85* were near that of the non-acclimated control. *CAP85* was resolved into a doublet of 85 kDa and a lower band of ≈80-84 kDa. This doublet was observed with both polyclonal and monoclonal antibodies. When protease inhibitors for the four major classes of proteases were included in the extraction buffer, the doublet pattern was not altered, nor was the intensity of the lower band reduced.

The protein used in antibody production and sequencing was composed of the doublet. Initial attempts to sequence the amino terminus from 2-D gel electroblots indicated a blocked amino terminus blockage. Amino acid composition analyses showed the presence of 0.4 mole % methionine (see Table 1), which made feasible the cleavage of *CAP85* with cyanogen bromide. From this procedure, we obtained three major peptides of 66, 55, and 50 kDa, which yielded the sequences shown in Figure 1. Peptide sequence analysis indicated homology of the 50 kDa peptide to Group 2 LEAs.

PCR cloning and cDNA library screening. The NH_2 terminus of *CAP85* is blocked. The peptide sequence of the CNBr peptides were used to construct degenerate synthetic oligonucleotides (55-A5Q = ATG AAG AAG AA(T or C) AA(C or T) AAG GG(C or T) GAG [SEQ ID NO. 5]; and 50-B3Q = TA(A or G) TC(A or G) TTC TTC TT(A or G) TCC TC(A or G) TG [SEQ ID NO. 6]) primers for the PCR amplification of cDNA derived from RNA extracted from 2 day cold acclimated leaf tissue. From this procedure, a 650 bp fragment (pcr733) was amplified, cloned, and labeled for use in RNA blot analyses. This fragment hybridized to a 2-0 to 2.2 kb RNA which was upregulated during cold acclimation. The deduced amino acid sequence of this fragment showed high homology to the amino acid sequences of the 55 kDa *CAP85* CNBr cleavage peptide, matching perfectly 35 of 37

identified residues (Figure 2). A screening of 100,000 plaques with the PCR fragment yielded 60 positives, of which the two largest clones were about 1.8 kb. Both clones hybridized to a strongly upregulated message during cold acclimation. There was a large increase in the level of message after one day of exposure to 5RC and the high levels of message were maintained over a 7-day exposure to 5RC. Upon return to 25RC, the message levels returned to that of non-acclimated plants and remained low throughout the deacclimation period. Sequencing demonstrated that the two clones were identical, except the smaller clone was missing approximately 200 bp from the 5Q end (Figures 3A-3B). Over 178 bp of the 3Q end were non-coding sequences. Sequence analysis indicated that the cDNA clones were identical to the PCR clone. At this stage, additional matches between the deduced protein sequence of the cDNAs and the peptide sequences became evident. The 55 kDa peptide matches the cDNA predicted sequence beginning at bp 109. A peptide fragment of this size can be deduced from the cDNA. One of the 66 kDa peptide sequences was identical to that of the 50 kDa peptide. Both were perfect matches to sequences in the cDNA beginning at bp 814. The second 66 kDa peptide sequence most closely matched a sequence near the carboxyl-terminus of the cDNA beginning at bp 1474. However, it clearly cannot be the source of the 66 kDa peptide.

A consensus translation start sequence is observed at the first ATG. The predicted size of the protein encoded by the cDNA clone is 61.5 kDa. This is roughly 72% of the estimated size of *CAP85* as determined by SDS-PAGE. However, the SDS-PAGE size estimates for many WSPs is usually greater than that predicted by DNA sequences. The predicted protein from the cDNA clone gives a calculated pI of 6.2, close to the estimated pI of 6.3 of *in vivo* produced *CAP85* as determined from 2-D gels, and the amino acid composition predicted by the clone is a close approximation to that determined from protein compositional analyses. The deduced protein is rich in charged residues (K 15%, D 12%, E 10%, and H 11%), but did not contain either cysteine or tryptophan.

Inspection of the cDNA sequence revealed similarity to the WSP gene family, or more specifically, to Group 2 LEAs. The gene showed a typical Group 2-like 11-residue lysine-rich repeat contained within a larger 22-amino acid sequence that was repeated 11 times within the clone (Figures 3A, 3B and 4). The repeats begin at around 300 bp into the 2.0 kb clone and continue to near the stop codon at bp 1640. The repeats are not contiguous but are separated irregularly by spans of 9 to 28 amino acids. Database searches indicated the highest homology to *Rab17*, which is a Group 2 LEA. Two other imperfect repeating motifs were also found in the primary structure of the deduced protein. Both were interspersed between

the lysine repeats. The longer repeat was present 4 times, while the shorter repeat was present 3 times (Figures 3A and 3B). *CAP85* differs from the Group 2 LEAs in that there is no serine cluster.

Blot of genomic DNA digests. Genomic Southern blots probed with either the PCR clone, pcr733, or the cDNA clone provided insight into the organization of the gene. Both probes produced the same pattern. The hybridization pattern appeared simple, which indicated that *CAP85* is present as a single gene or a small gene family.

Increased message and protein levels of *CAP85* during cold and water stress. When spinach was subjected to a water stress, there was a dramatic increase in the message levels. The levels of message remained elevated throughout the desiccation stress period. Once the plant was returned to a normal hydrated state, the message levels decreased to non-stressed levels within one day. Spinach also showed an increased abundance of *CAP85* protein in response to a water stress. In contrast, the protein levels remained high even after return to a normal hydration state. The protein turn-over rate appeared to be slower than that of the message, as elevated protein levels were evident 24 hours following the return to non-stress conditions. Also, while changes in the protein levels were apparent, the changes in the message abundance were far more pronounced.

The distribution of *CAP85* protein and message. The distribution of the protein and the message in spinach seedlings was determined. Protein and RNA were extracted from various tissues from 2 day cold acclimated plants. Immunodetectable protein was found in the leaf, cotyledon, hypocotyl, and root. *CAP85* protein was also detected in seeds and pollen. Similarly, the *CAP85* message was present in leaf, petiole, and root tissues during cold acclimation. Cell fractionation studies with leaf and hypocotyl tissue showed that *CAP85* was present in the cytosol, and possibly in the endoplasmic reticulum and chloroplast.

Boiling solubility of *CAP85*. Proteins were extracted from 2 day cold acclimated leaf tissues and subjected to 2 and 10 minute boiling treatments. Protein blot analyses confirmed that *CAP85* is not rendered insoluble by boiling.

Example 2 — Amplification of *CAP85* cDNA

Amplification of the cDNA to obtain a *CAP85* probe selected for a gene with an orientation which placed the 55-A5Q sequence before that of the 50-B3Q sequence. In screening the cDNA library with pcr733, we selected for the gene with the 55-A5Q⇒50-B3Q arrangement. Both pcr733 and the cDNA clone hybridize, under high stringency, to the gene containing the 55-A5Q⇒50-B3Q orientation, and not the B⇒A orientation. Even if the two

genes enc de closely related proteins, the bias of the PCR and cDNA clones would select for only one member of the *CAP85* doublet. The match of the cDNA clone to the 55 kDa peptide sequence from *CAP85* establishes the linkage of this gene to a protein that is associated with the cold acclimation process. In addition, the amino acid composition of the predicted protein and that of *CAP85* are similar, as are the observed and the estimated isoelectric points. Finally, the influences of low temperature and water stress on the abundance of the transcript are consistent with the accumulation of *CAP85* in response to these conditions.

Example 3 — Secondary and Tertiary Structure of *CAP85*

For secondary and tertiary structure of the protein, an α-helical structure in the 22-mer lysine repeating motif of *CAP85* is suggested. In plotting a 3.6 amino acids/turn α-helix, beginning with the first member of the motif, we observe four distinct faces. The first face is primarily composed of acidic residues (D_{12}, E_5, D_{16}), while the second face is composed of basic residues (K_6, K_{13}, K_{17}). The third face is mostly non-polar residues with adjacent (E_3, K_7) acidic and basic residues, which can form a salt bridge. Additional salt bridge combinations can act to stabilize the α-helical structure. The final face is a mixture of acid, basic, non-polar, and polar residues. The repeating motif, from residue 1 to 18, forms four complete cycles ending at residue P_{19}. Proline is associated with loop structures. The glycine immediately following P_{19} further favors that this part of the peptide is a turn or loop region. The 11 repeating motifs in the α-helical conformation can join in helix-helix associations either along the non-polar regions or in anti-parallel arrangements along the acidic and basic faces. These associations along the non-polar regions permit the charged residues to be free to form interactions with charged cellular components. Such helices can interact with phospholipid head groups of the membrane. In such associations, the formation of ionic interactions between the charged phospholipid head groups and *CAP85* can function to stabilize the membrane both during water loss due to dehydration and also exposure to low temperature by acting as a reinforcing lattice network. A coating of *CAP85* on the inner face of the plasma membrane can provide a matrix able to stabilize membrane structure during extreme loss of cellular water and volume. These highly hydrophilic proteins participate in adaptive mechanisms in plants during cold and water stress conditions.

Example 4 — Cloning of *CAP160*

Antibody screening was used to isolate cDNAs for *CAP160*. Sixty clones were selected from initial screenings of the same cDNA library used to isolate *CAP85*. Several of these clones were used for a RNA blot screening to verify that the cDNA corresponded to a gene that was upregulated during cold acclimation. The clones with the largest inserts were further characterized by protein blot analysis of the fusion protein in *E. coli* lysates. Two clones expressed a fusion protein that appeared to be slightly larger than authentic *CAP160* obtained from spinach leaf tissue. These clones, along with another lacking 200 bp from the NH_2-terminus, were used for sequencing. From sequence analysis, the cDNAs for *CAP160* appear to contain full coding sequence for the protein. We were also able to match the deduced amino acid sequence with protein sequence information obtained from sequencing CNBr cleavage products of purified *CAP160*, firmly establishing the identify of the cDNAs.

Like *CAP85*, *CAP160* has repeating motifs. However, the pattern and sequence of the repeating elements are very different from those of *CAP85*. The repeats are fewer and larger in *CAP160*. No significant sequence similarity at the amino acid level appears between *CAP160* and *CAP85*. Only in the carboxyl-termini of both proteins is there significant homology over a short 10-residue region that happens to encompass a lysine repeat of *CAP85*. Evidence that the cDNAs encode *CAP160* include the antibody reactivity, low temperature upregulation, the size of product at roughly 160 kDa by SDS-PAGE, and the cDNA sequence closely matches the amino acid sequence determined from CNBr cleavage fragments of purified *CAP160*.

We have screened the genomic library for *CAP160* clones. Five clones have been isolated and confirmed by PCR with three different sets of *CAP160* primers. The clones range from 4 to perhaps greater than 6 kb.

Example 5 — Insertion of Cold or Drought Tolerance Genes Into Plants

One aspect of the subject invention is the transformation of plants with genes encoding a cold acclimation protein as disclosed herein. The transformed plants are resistant to damage by extreme cold temperatures, freezing, or drought. The transformed plants can be monocots or dicots and, in a preferred embodiment, would be citrus. When transforming monocots, it may be most advantageous to transform embryogenic cells or tissue using DNA bound to high-velocity microprojectiles as a means of delivering it to the embryogenic cells. See for example, Fromm, M.E., F. Morrish, C. Armstrong, R. Williams, J. Thomas, T.M. Klein [1990] "Inheritance of expression of chimeric genes in the progeny of transgenic maize plants," *Bio/Technology* 8:833-839.

Genes encoding cold acclimation proteins, as disclosed herein, can be inserted into plant cells using a variety of techniques which are well known in the art. For example, a large number of cloning vectors comprising a replication system in *E. coli* and a marker that permits selection of the transformed cells are available for preparation for the insertion of foreign genes into higher plants. The vectors comprise, for example, pBR322, pUC series, M13mp series, pACYC184, and the like. Accordingly, the sequence encoding a cold acclimation protein can be inserted into the vector at a suitable restriction site. The resulting plasmid is used for transformation into *E. coli*. The *E. coli* cells are cultivated in a suitable nutrient medium, then harvested and lysed. The plasmid is recovered. Sequence analysis, restriction analysis, electrophoresis, and other biochemical-molecular biological methods are generally carried out as methods of analysis. After each manipulation, the DNA sequence used can be cleaved and joined to the next DNA sequence. Each plasmid sequence can be cloned in the same or other plasmids. Depending on the method of inserting desired genes into the plant, other DNA sequences may be necessary. If, for example, the Ti or Ri plasmid is used for the transformation of the plant cell, then at least the right border, but often the right and the left border of the Ti or Ri plasmid T-DNA, has to be joined as the flanking region of the genes to be inserted.

The use of T-DNA for the transformation of plant cells has been intensively researched and sufficiently described in EP 120 516; Hoekema (1985) In: *The Binary Plant Vector System*, Offset-durkkerij Kanters B.V., Alblasserdam, Chapter 5; Fraley *et al.*, *Crit. Rev. Plant Sci.* 4:1-46; and An *et al.* (1985) *EMBO J.* 4:277-287.

Once the inserted DNA has been integrated in the genome, it is relatively stable there and, as a rule, does not come out again. It normally contains a selection marker that confers on the transformed plant cells resistance to a biocide or an antibiotic, such as kanamycin, G 418, bleomycin, hygromycin, or chloramphenicol, *inter alia*. The individually employed marker should accordingly permit the selection of transformed cells rather than cells that do not contain the inserted DNA.

A large number of techniques are available for inserting DNA into a plant host cell. Those techniques include transformation with T-DNA using *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes* as transformation agent, fusion, injection, electroporation, or the use of high-velocity microprojectiles, as well as other possible methods. If agrobacteria are used for the transformation, the DNA to be inserted has to be cloned into special plasmids, namely either into an intermediate vector or into a binary vector. The intermediate vectors can be integrated into the Ti or Ri plasmid by homologous recombination owing to sequences

that are homologous to sequences in the T-DNA. The Ti or Ri plasmid also comprises the *vir* region necessary for the transfer of the T-DNA. Intermediate vectors cannot replicate themselves in agrobacteria. The intermediate vector can be transferred into *Agrobacterium tumefaciens* by means of a helper plasmid (conjugation). Binary vectors can replicate themselves both in *E. coli* and in agrobacteria. They comprise a selection marker gene and a linker or polylinker which are framed by the right and left T-DNA border regions. They can be transformed directly into agrobacteria (Holsters *et al.* [1978] *Mol. Gen. Genet.* 163:181-187). The agrobacterium used as host cell is to comprise a plasmid carrying a *vir* region. The *vir* region is necessary for the transfer of the T-DNA into the plant cell. Additional T-DNA may be contained. The bacterium so transformed is used for the transformation of plant cells. Plant explants can advantageously be cultivated with *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes* for the transfer of the DNA into the plant cell. Alternatively, DNA may be introduced into plant cell suspensions, embryogenic cells, or other embryogenic tissue by one of the methods noted above. Whole plants can then be regenerated from the treated plant material (for example, pieces of leaf, segments of stalk, roots, but also protoplasts or suspension-cultivated cells) in a suitable medium, which may contain antibiotics or biocides for selection. The plants so obtained can then be tested for the presence of the inserted DNA. No special demands except for a selectable or screenable marker are made of the plasmids in the case of injection and electroporation. It is possible to use ordinary plasmids, such as, for example, pUC derivatives.

The transformed cells grow inside the plants in the usual manner. They can form germ cells and transmit the transformed trait(s) to progeny plants. Such plants can be grown in the normal manner and crossed with plants that have the same transformed hereditary factors or other hereditary factors. The resulting hybrid individuals have the corresponding phenotypic properties.

A variety of plants have been genetically transformed according to the above-described methods. These plants include alfalfa, apple, asparagus, broccoli, cabbage, carrot, cauliflower, celery, corn, cotton, cranberry, cucumber, eggplant, flax, grape, horseradish, kiwi fruit, lettuce, muskmelon, oilseed rape, papaya, pea, pepper, plum, poplar, potato, raspberry, rice, rye, soybean, spruce, strawberry, sugarbeet, sugarcane, sunflower, sweet potato, tobacco, tomato, walnut, and wheat (Gasser, C.S., R.T. Fraley [1992] *Scientific American* June:62-69). Other transgenic plants of interest which can be produced are plants included in the family Solanaceae, as well as citrus.

Example 6 — Enhancement of Cold Tolerance in Microorganisms

The inability of *Escherichia coli* to survive a freeze/thaw cycle is well known. We demonstrated that *CAP160* can be expressed in a microorganism and, advantageously, alter its cryotolerance. *Escherichia coli* were transformed with a gene expressing a *CAP160* DNA β-galactosidase fusion protein according to methods well known in the art. The survival rate of the transformed *E. coli* cells following a freeze/thaw cycle was compared against cells containing pBluescript plasmid without an insert (wild-type).

XL1-Blue cells containing a pBluescript plasmid with the *CAP160* DNA, or without an insert, were grown to midlog phase in an LB/AMP medium at 37RC. Fusion protein expression was induced by addition of IPTG to the culture medium (1 or 10 mM final concentration). The cultures were allowed to incubate for an additional 30 minutes at 37RC following the addition of IPTG. Aliquots of cells in culture tubes were then equilibrated to —5RC in a controlled temperature bath and seeded with a chip of sterile ice to initiate freezing of the culture medium. Freezing of the 2 ml culture medium equilibrated to —5RC was rapid with the transition from liquid to solid phase occurring within a minute. After one hour at —5RC, frozen cells were either maintained at —5RC, or transferred to —20RC. After one hour at —20RC, aliquots of the cells were transferred to —80RC. The rate of cooling from —5RC to —20RC and —80RC was not determined. After 16 hours, the cultures were thawed at 4RC, and survival was determined by dilution series plate counts following overnight growth on LB/AMP agar plates at 37RC. Unfrozen cells kept at 4RC served as the control. The data, as shown in Table 2, below, are expressed as the percent survival relative to the unfrozen control. The values are the mean ± SE of nine separate experiments.

Table 2. Survival of recombinant *Escherichia coli* following a freeze/thaw stress

	Temperature (RC)		
	—5	—20	—80
pBluescript only			
—IPTG	11 ± 4	4 ± 2	1 ± 1
+IPTG	10 ± 2	3 ± 1	1 ± 1
pBluescript w/*CAP160*			
—IPTG	27 ± 8	5 ± 2	1 ± 1
+IPTG	31 ± 9	6 ± 1	2 ± 1

After freezing at −5RC, the survival rate for the cells expressing *CAP160* was roughly three-fold higher than the wild-type cells containing pBluescript plasmid only. Induction with IPTG only slightly altered the survival rate in the *CAP160* producing cells and had no effect on the others. At temperatures below −5RC, survival of *CAP160* cells was slightly higher than that of the cells containing pBluescript only.

To determine the equilibrium freezing LT_{50} (killing temperature for 50% of the cells), cultures induced with IPTG were cooled at 2RC per hour to −20RC following the initiation of freezing with a sterile ice chip at −2RC. Survival of frozen cells was determined after exposure to −2, −4, −6, −8, −10, −15, or −20RC and thawing overnight at 4RC. Cells containing the pBluescript plasmid without an insert lost viability rapidly between −2R and −4RC (Figure 5) and yielded an estimated LT_{50} of −3RC. The cells expressing the *CAP160* fusion protein showed substantially greater survival following freezing. The estimated LT_{50} of these cells was about −6RC, and between −6RC and −20RC, their survival rate was between four and seven fold higher than wild-type cells. Clearly, expressing the *CAP160* fusion protein enhanced the ability of XL1-Blue cells to survive a freeze/thaw stress.

Freezing is much more deleterious to *E. coli* than chilling and/or supercooling. That *CAP160* was protecting against freeze/thaw stress and not against chilling stress was indicated by the near 100% survival rate of cells supercooled to −10RC and warmed without freezing (Figure 5).

Other transgenic microorganisms can be produced by methods that are well known and which can be conducted by a person of ordinary skill in the art. These other organisms include other bacteria as well as eukaryotic microorganisms such as yeast.

Homology searches indicate that *CAP160*, in its entirety, is a novel protein whose biological function cannot be identified by shared homology with known proteins, including antifreeze and ice nucleation proteins that function to alter freezing of water. Thus, *CAP160* represents a new class of intracellular low temperature stress proteins with a function linked to enhanced freezing tolerance mechanisms in microorganisms.

Example 7 — Construction of Chimeric Genes

Any number of structural chimeric cold acclimation genes can be constructed using readily available promoters and polyadenylation signals. One example is to use the cauliflower mosaic virus 35S promoter, which is a strong constitutive promoter, to drive transcription of the cold acclimation gene, and a nopaline synthase polyadenylation signal to ensure the RNA is properly processed and translated into a functional protein (Shah *et al.*

[1986] *Science* 233:478-481). Transformed kanamycin resistant cells can be regenerated into plants and tested for enhanced freezing tolerance. Plants showing enhanced cold tolerance can be analyzed to show that the introduced cold acclimation genes are responsible for the change in hardiness. Promoters for inducible genes can also be used in chimeric cold acclimation gene constructs. Natural promoters for these genes also can be used. This allows the cold acclimation genes to be activated when needed to enhance freezing tolerance at specific times. Other inducible promoters can also be used.

In order to explore the prospect that *CAP160* and *CAP85* form a complex that requires both proteins for optimum function, plants expressing both spinach proteins can be produced. Two ways to achieve this are to transform transgenic plants already expressing one of the spinach proteins with a construct from the second gene and to make constructs that contain both cDNA sequences that can be co-expressed in transformed plants or, as above, transferred sequentially. In the former case, a second selective media can be employed using the *bar* gene (Vasil *et al.* [1992] *Bio/Technology* 10:667-674).

It should be understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and the scope of the appended claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: UNIVERSITY OF FLORIDA

(ii) TITLE OF INVENTION: Novel Genes, Polypeptides, and Compositions for Cold Tolerance and Drought Resistance in Plants

(iii) NUMBER OF SEQUENCES: 6

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Saliwanchik & Saliwanchik
(B) STREET: 2421 N.W. 41st Street, Suite A-1
(C) CITY: Gainesville
(D) STATE: FL
(E) COUNTRY: USA
(F) ZIP: 32606

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: US
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: WHITLOCK, TED W.
(B) REGISTRATION NUMBER: 36,965
(C) REFERENCE/DOCKET NUMBER: UF/S&S-109

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 904-375-8100
(B) TELEFAX: 904-372-5800

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1813 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CACGAGCTAA TTTGTTGTAA TCAAGCAATA ACAATGGCTG ATGAAAGGAA CACTTATGGG 60

GGACCCGCAC CATCTATGGA GACCACTGAT CGTGGTATGT TTGATTTCAT GAAGAAGAAC 120

AACAAGGGAG AGGATCACAA GCCATCCGAG GCCGATGTGA TTGCCTCCGG CGGTATCGGA 180

AAGTTGCCCG TCTCCGAACC TGCTCATTAT GACCATGATG ACAAGGAACA TGTTGGACTC 240

CTTGAGAAAA AACATATTGG ACTTGTTGAG CAATTCCATC GTTCTGATCA CGCTTCCGAC 300

GAAAGACATC ATGATGAAGA GCAAAACAAA GGTGGTGTCT TCGGAAAAAT CAAGGAGAAG 360

CTCCCCGGTC AGCATGATTC GGATACTACC ACACATACAC AACAATTATA CCCTGCTTCT 420

GATCATAACT ACAACACCCA CCATGTCCAC CAAGACGATG AAAAGAAGGA CAACATCCTT 480

GACAAAATCA AGGATAAGCT TCCCGGGAAA CATGAAGATA AGAAGCAAGA CTATCACCAG 540

CACCAAGAGG AGGAAAAGAA GGGAGGAGCC CTTGACAAAA TCAAGGACAA GCTGCCCGGT 600

CAGGGTAATG CTGGACACAC GCAGCAATTA TACCCTGCCC CTGATCATAA CTACAACACA 660

CACCATGTCC ACCAAGACGA GGAAAACAAG GATAGTGTCT TAGACAAAAT CAAGGATAAG 720

CTGCCCGGAC AACATGAAGA TAAGAAGAAT GACTATCACC ACCACCAAGA GGAGGAAAAG 780

AAGGATAGTG TCCTAGACAA AATCAAGGAT AAGATGTCCG GGCAGCATGA AGATAAGAAG 840

AATGACTATC ACCACCACCA AGAGGAGGAA AAGAAGGGAG GAGTCCTTGA CAAAATCAAG 900

GACAAGTTGC CTGGTCAACA TGATGCAGAC ACTGCCAGAC ACACGCAGCA ACTATACCCT 960

GCTGCTGATC ATAACTACAA CACACACCAT GTCCACCAAG ATGAGGAAAA CAAGGATAGC 1020

GTCCTTGACA AAATCAAGGA CAAACTACCC GGACAACATG ATGATAAGGC TGCATACTCG 1080

CAACATGACC ACCACAAGCA CCACCAAGAG GAGGAAAACA AGGGTGGAGT CCTCGACAAA 1140

ATCAAGGACA AACTGCCTGG TGTCTACATG GTGGTCAAAC ATGATGGTGA TATTGTCGAA 1200

CACACGCAAC AATTATACCC TGCTCCTGAT CATAACTACA ACACTCACTA TGTCCATGAA 1260

GACGAGAAAA AGAAGGATAG TGTCCTAGAC AAAATCAAGG ACAAGTTACC CGGACAACAT 1320

GAGGAAAAGG CAGCAGCATA CTCTGAGCCA TCATATGATT CACACCCTAC ACCTGCAAAG 1380

CATCATGATT ATTTCCCCCA AGAGGAGGAA AAGAAAGGTG GTGTCATGGA CAAAATTAAG 1440

GACAAGCTTT CCGGCCAACA TAAAGATAAG GCCGACGAGC ATGAGTTGGT TGCTCCGTTG 1500

GTGACAGTCG AACCACATTC TGAGGGTGAT AAGGAAAAGA AGGGGTTCTT GGAGAAGATT 1560

AAGGACAAAA TCCCCGGCCT CCACTCCAAG AATGATGCTG AAGAGAAGAA GACCCATGAG 1620

GAGAAAAAAG AGGGATACTA AACTTAACTA ATAAATATCT ACGTATATTA TGTTCAATAA 1680

GATCGAATTA GTTGCTTTTT TTAGGTTGAT GTGTTTTTCT TGATCAATGC TTTGTGTAAT 1740

TTGAGTCCAA ACTGTGGGGT TTTGATGTCA GTGTTTTTTT CATGACGATG AATATGCAAT 1800

TATGTGTGTA TGG 1813

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 535 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Ala Asp Glu Arg Asn Thr Tyr Gly Gly Pro Ala Pro Ser Met Glu
1 5 10 15

Thr Thr Asp Arg Gly Met Phe Asp Phe Met Lys Lys Asn Asn Lys Gly
20 25 30

Glu Asp His Lys Pro Ser Glu Ala Asp Val Ile Ala Ser Gly Gly Ile
35 40 45

Gly Lys Leu Pro Val Ser Glu Pro Ala His Tyr Asp His Asp Asp Lys
50 55 60

Glu His Val Gly Leu Leu Glu Lys Lys His Ile Gly Leu Val Glu Gln
65 70 75 80

Phe His Arg Ser Asp His Ala Ser Asp Glu Arg His His Asp Glu Glu
85 90 95

Gln Asn Lys Gly Gly Val Phe Gly Lys Ile Lys Glu Lys Leu Pro Gly
100 105 110

Gln His Asp Ser Asp Thr Thr Thr His Thr Gln Gln Leu Tyr Pro Ala
115 120 125

Ser Asp His Asn Tyr Asn Thr His His Val His Gln Asp Asp Glu Lys
130 135 140

Lys Asp Asn Ile Leu Asp Lys Ile Lys Asp Lys Leu Pro Gly Lys His
145 150 155 160

Glu Asp Lys Lys Gln Asp Tyr His Gln His Gln Glu Glu Glu Lys Lys
165 170 175

Gly Gly Ala Leu Asp Lys Ile Lys Asp Lys Leu Pro Gly Gln Gly Asn
180 185 190

Ala Gly His Thr Gln Gln Leu Tyr Pro Ala Pro Asp His Asn Tyr Asn
195 200 205

Thr His His Val His Gln Asp Glu Glu Asn Lys Asp Ser Val Leu Asp
210 215 220

Lys Ile Lys Asp Lys Leu Pro Gly Gln His Glu Asp Lys Lys Asn Asp
225 230 235 240

Tyr His His His Gln Glu Glu Glu Lys Lys Asp Ser Val Leu Asp Lys
245 250 255

Ile Lys Asp Lys Met Ser Gly Gln His Glu Asp Lys Lys Asn Asp Tyr
260 265 270

His His His Gln Glu Glu Glu Lys Lys Gly Gly Val Leu Asp Lys Ile
275 280 285

Lys Asp Lys Leu Pro Gly Gln His Asp Ala Asp Thr Ala Arg His Thr
290 295 300

Gln Gln Leu Tyr Pro Ala Ala Asp His Asn Tyr Asn Thr His His Val
305 310 315 320

His Gln Asp Glu Glu Asn Lys Asp Ser Val Leu Asp Lys Ile Lys Asp
325 330 335

Lys Leu Pro Gly Gln His Asp Asp Lys Ala Ala Tyr Ser Gln His Asp
340 345 350

His His Lys His His Gln Glu Glu Glu Asn Lys Gly Gly Val Leu Asp
355 360 365

Lys Ile Lys Asp Lys Leu Pro Gly Val Tyr Met Val Val Lys His Asp
370 375 380

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Gly Asp Ile Val Glu His Thr Gln Gln Leu Tyr Pro Ala Pro Asp His
385                 390                 395                 400

Asn Tyr Asn Thr His Tyr Val His Glu Asp Glu Lys Lys Lys Asp Ser
                405                 410                 415

Val Leu Asp Lys Ile Lys Asp Lys Leu Pro Gly Gln His Glu Glu Lys
            420                 425                 430

Ala Ala Ala Tyr Ser Glu Pro Ser Tyr Asp Ser His Pro Thr Pro Ala
        435                 440                 445

Lys His His Asp Tyr Phe Pro Gln Glu Glu Glu Lys Lys Gly Gly Val
    450                 455                 460

Met Asp Lys Ile Lys Asp Lys Leu Ser Gly Gln His Lys Asp Lys Ala
465                 470                 475                 480

Asp Glu His Glu Leu Val Ala Pro Leu Val Thr Val Glu Pro His Ser
                485                 490                 495

Glu Gly Asp Lys Glu Lys Lys Gly Phe Leu Glu Lys Ile Lys Asp Lys
            500                 505                 510

Ile Pro Gly Leu His Ser Lys Asn Asp Ala Glu Glu Lys Lys Thr His
        515                 520                 525

Glu Glu Lys Lys Glu Gly Tyr
    530                 535
```

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2720 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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GCACAAGGTT ATAACCTCCT ATTTATTTCC AATTCAATTT GTATCTCATT TTATATAAAT      60

AGTATTTTCA ATTAAAAAAA CCAAGAATTC AAAATGGAAC ACCCTAGCGG TCACACGCGT     120

CACACTACTC ATGTAGAAGA TGATCTTCAA GATGCTTCCA TACAAACTGG TCATAATGAA     180

GATGAGAAGC CTGAGAAGAA AACAATGATG ATGAAAGTAA AGGCGAAAGC AAGGAAGATT     240

AGAGACAGTA TTAAGAATGT TGGACATAGT CATGATCATG ATCATGATCA CGATGAGAAT     300

GACGACGATG ATGACGAGGA GGAGGAAGTT GAGATGGATA TGGACTCTGA AATCCAAGGC     360

ACTCATACTG CTCAAACCGG CACACCAGGG GAGGAAGTTA CAAGGCAGAA GCTGCATGAA     420

CCAAAACTGG TAGAAAGAAC AATAGGTGAA GATATCCAAG TTCGGAACAG ACTAGGTGAT     480

TATCAGACAT TTGATCCTAC TTCTGAAACA TTCACTCCTG GACATGATCA GACCTTAGGT     540

TGGTCGAGGA CTGATACCGG AAAGCCAAAA GAGTACGGTG GAAGTCATAG TACTGAAGCT     600

TCTGATAAAG AGATGAATGC AGCAGCTCCT GTAAATCTTG GAGGTGTTGT TGTTGGTTGT     660

GACCATCAGG TCCCGAAAGA TGTAGGGGAA GATAGTCATT CTGCTAATTA TCAGTCTGAA     720

GTCATTGAAC CAACAGTTAC TGGATTCGAA TTCCCGGTTG CTCAAAGTCA TTCACCAGCA     780
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AATAAGCCAC AGGGCGATTT TCAGACCTTT AATCCTAGGA CTGATACTGA AAGGCTAAAC 840

AAGTCAGTGG AAATGTTCAA TGAATCGAAT AATGCACCAA TATCAGCCCA TTCAGGACAT 900

GAATCACATG AAAGAACTGC AATGGAAGGG GTGGTTGATG CCCCGGGAAA CAAGACAGAA 960

GGCGATTATC AGACCTTTGA TCCTAAGTCT ACAAGCTATG TTCCCGGACA AGAAGAGACC 1020

TTGGGTTGGT CTAGAACTGA CACCGGAGGG CTAAACAAGT CCGAGGAACT TTCCAATCTA 1080

TCGAACAACA CATCAACTGA AACTCATTCA GGTGATGAAG AAACAAGGAT TATTCAAATC 1140

CTGAATCAGA TGGATTTAAT GAACGTTAAC GAAGAATCGC AGCAGAAACC AACAGCACCA 1200

GATGATTCTC ACCTGAACAA AACAGAACAT CATAATCCAC CAGATGAGAA AATCTCAACT 1260

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CAAGCTACTA CTACTCCAGC CACTGATGGT AATAGCTACA CCGGAATGAT ATCAAATGCG 1380

GCTGCAATGG TGGCTGATAA GGCAATGCTA GCCACTAGTG CTGTTACATC AAAGCTAGGG 1440

TATGGTGGGC CATCCACCGG GCCTACTAGT CCTGATCAGC AGCACTCAAC AACTGATGTA 1500

ACTTCAGAAA TGCACGACAA CAACCCGTCA GATAAGCCCG TGGGTACTAC TTATGGGGAG 1560

AGAATGTCGA GTGCCACAGC TGTTGTTACT GATAAAGCTA TACAAGCCAA GGATGTTGTA 1620

GCCACTAAGC TAGGCTATGG TGGCAATCCT GATCAGCAGC ACTCCACTGA TGTGACTTCA 1680

GAAATGCATG ATAACCCGTC AGAGCAGCCC GTGGGTACTA CCTACGGGGA GAAAATATCG 1740

AGTGCCACGA CTGTTGTTAC TGATAAGGCT ATACAAGCCA AGGATGTTGT AGCCGCTAAG 1800

CTGGGCTATG GCGGGCCGTC CACCGGGCCA TCCACTGGGC CTATTACCGG ACCATCCAAG 1860

GGGCCTATTA CCGGGCCATC CACCGGGCCT ATTACATGGG GGGATAAGGG AGTGGCAGTG 1920

AAGGAGTATT TGGTAGAGAA ACTGAAGCCC GGTGAAGATG ACAAGGCGTT GTCCGAGGTT 1980

ATAACTGAAG CCTTGCCTTC GCCCTTACAC AAACCGAAGG AGGAGGGTGT GACTATAATA 2040

GGGAGAGTTG CAGAGCCTAA AGAGGTGGTG CAAATGATTG ATCATATTGA GGAGAAGAAT 2100

GATGATGGTA TAGTGATGGG TGAAGATGAC AAGGCAGTGT TCGAGGCTGT AGTTGGGAAG 2160

GTAGGGGGAG GGGACGAGGT TGCGGAAAGG CTTGGTTGGG GGGAGGAGAA GAAGGAGGAT 2220

GGTAGTGATA ATGGTGGTGC AGGGGTGGTT AGTCCAGGGA AGGGTGTAAT GGAAAGGATT 2280

AAAGATGCTG CTAGTGGGTG GTTTCAGAGT AGTGATGACT TTCCGTCTCA GGATACCGGT 2340

ACCCGTACCC GTCATATCAC ACAAGGAACG GAAAGTTTTC CCATTTCTAG CATGGAAAGT 2400

GAGCAGAAAA GAATCGGTGG TGCAGCGTCT CTTTAGTAAT TTGAGCTTAG ATATGTGTTC 2460

CGGGTCTGGA AGTGGAACTG GGGGATGATC TGGAAGTACC TTGCCCTGTC AAGTAGAGCA 2520

AACGTTAGCC CCACTTTACG ACCCAGTACA ATATGTTGTA TTCCCGGATT TTTTTTATTT 2580

GTGATGTGTA AACAATAGCA GTTTGCTTCA GGAGTAATGC TGCTAGCTTG CTAGGAATGT 2640

ACTTCAAATT AACAACTGTT GTAATTTTGT AAATAAAGAA CTGGACACTT TCCAGAGTTT 2700

GTGAAAAAAA AAAAAAAAAA 2720

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 802 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

```
Thr Arg Ala Asn Ser Leu Ala Thr Glu Asp Phe Arg Met Cys Cys Ala
1               5                  10                  15

Pro Arg Phe Ser Glu Gln Met Glu His Pro Ser Gly His Thr Arg His
            20                  25                  30

Thr Thr His Val Glu Asp Asp Leu Gln Asp Ala Ser Ile Gln Thr Gly
        35                  40                  45

His Asn Glu Asp Glu Lys Pro Glu Lys Lys Thr Met Met Met Lys Val
    50                  55                  60

Lys Ala Lys Ala Arg Lys Ile Arg Asp Ser Ile Lys Asn Val Gly His
65                  70                  75                  80

Ser His Asp His Asp His Asp His Asp Glu Asn Asp Asp Asp Asp Asp
                85                  90                  95

Glu Glu Glu Glu Val Glu Met Asp Met Asp Ser Glu Ile Gln Gly Thr
            100                 105                 110

His Thr Ala Gln Thr Gly Thr Pro Gly Glu Glu Val Thr Arg Gln Lys
        115                 120                 125

Leu His Glu Pro Lys Leu Val Glu Arg Thr Ile Gly Glu Asp Ile Gln
    130                 135                 140

Val Arg Asn Arg Leu Gly Asp Tyr Gln Thr Phe Asp Pro Thr Ser Glu
145                 150                 155                 160

Thr Phe Thr Pro Gly His Asp Gln Thr Leu Gly Trp Ser Arg Thr Asp
                165                 170                 175

Thr Gly Lys Pro Lys Glu Tyr Gly Gly Ser His Ser Thr Glu Ala Ser
            180                 185                 190

Asp Lys Glu Met Asn Ala Ala Ala Pro Val Asn Leu Gly Gly Val Val
        195                 200                 205

Val Gly Cys Asp His Gln Val Pro Lys Asp Val Gly Glu Asp Ser His
    210                 215                 220

Ser Ala Asn Tyr Gln Ser Glu Val Ile Glu Pro Thr Val Thr Gly Phe
225                 230                 235                 240

Glu Phe Pro Val Ala Gln Ser His Ser Pro Ala Asn Lys Pro Gln Gly
                245                 250                 255

Asp Phe Gln Thr Phe Asn Pro Arg Thr Asp Thr Glu Arg Leu Asn Lys
            260                 265                 270

Ser Val Glu Met Phe Asn Glu Ser Asn Asn Ala Pro Ile Ser Ala His
        275                 280                 285

Ser Gly His Glu Ser His Glu Arg Thr Ala Met Glu Gly Val Val Asp
    290                 295                 300
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Ala Pro Gly Asn Lys Thr Glu Gly Asp Tyr Gln Thr Phe Asp Pro Lys
305                 310                 315                 320

Ser Thr Ser Tyr Val Pro Gly Gln Glu Glu Thr Leu Gly Trp Ser Arg
                325                 330                 335

Thr Asp Thr Gly Gly Leu Asn Lys Ser Glu Glu Leu Ser Asn Leu Ser
            340                 345                 350

Asn Asn Thr Ser Thr Glu Thr His Ser Gly Asp Glu Glu Thr Arg Ile
        355                 360                 365

Ile Gln Ile Leu Asn Gln Met Asp Leu Met Asn Val Asn Glu Glu Ser
    370                 375                 380

Gln Gln Lys Pro Thr Ala Pro Asp Asp Ser His Leu Asn Lys Thr Glu
385                 390                 395                 400

His His Asn Pro Pro Asp Glu Lys Ile Ser Thr Glu Ser His His Asp
                405                 410                 415

Gln Phe Phe Ala Lys Pro Asp Thr Ser Glu Thr Gly Pro Val Val Gln
            420                 425                 430

Ala Thr Thr Thr Pro Ala Thr Asp Gly Asn Ser Tyr Thr Gly Met Ile
        435                 440                 445

Ser Asn Ala Ala Ala Met Val Ala Asp Lys Ala Met Leu Ala Thr Ser
    450                 455                 460

Ala Val Thr Ser Lys Leu Gly Tyr Gly Gly Pro Ser Thr Gly Pro Thr
465                 470                 475                 480

Ser Pro Asp Gln Gln His Ser Thr Thr Asp Val Thr Ser Glu Met His
                485                 490                 495

Asp Asn Asn Pro Ser Asp Lys Pro Val Gly Thr Thr Tyr Gly Glu Arg
            500                 505                 510

Met Ser Ser Ala Thr Ala Val Val Thr Asp Lys Ala Ile Gln Ala Lys
        515                 520                 525

Asp Val Val Ala Thr Lys Leu Gly Tyr Gly Gly Asn Pro Asp Gln Gln
    530                 535                 540

His Ser Thr Asp Val Thr Ser Glu Met His Asp Asn Pro Ser Glu Gln
545                 550                 555                 560

Pro Val Gly Thr Thr Tyr Gly Glu Lys Ile Ser Ser Ala Thr Thr Val
                565                 570                 575

Val Thr Asp Lys Ala Ile Gln Ala Lys Asp Val Val Ala Ala Lys Leu
            580                 585                 590

Gly Tyr Gly Gly Pro Ser Thr Gly Pro Ser Thr Gly Pro Ile Thr Gly
        595                 600                 605

Pro Ser Lys Gly Pro Ile Thr Gly Pro Ser Thr Gly Pro Ile Thr Trp
    610                 615                 620

Gly Asp Lys Gly Val Ala Val Lys Glu Tyr Leu Val Glu Lys Leu Lys
625                 630                 635                 640

Pro Gly Glu Asp Asp Lys Ala Leu Ser Glu Val Ile Thr Glu Ala Leu
                645                 650                 655

Pro Ser Pro Leu His Lys Pro Lys Glu Glu Gly Val Thr Ile Ile Gly
            660                 665                 670
```

Arg Val Ala Glu Pro Lys Glu Val Val Gln Met Ile Asp His Ile Glu
675 680 685

Glu Lys Asn Asp Asp Gly Ile Val Met Gly Glu Asp Asp Lys Ala Val
690 695 700

Phe Glu Ala Val Val Gly Lys Val Gly Gly Gly Asp Glu Val Ala Glu
705 710 715 720

Arg Leu Gly Trp Gly Glu Glu Lys Lys Glu Asp Gly Ser Asp Asn Gly
725 730 735

Gly Ala Gly Val Val Ser Pro Gly Lys Gly Val Met Glu Arg Ile Lys
740 745 750

Asp Ala Ala Ser Gly Trp Phe Gln Ser Ser Asp Asp Phe Pro Ser Gln
755 760 765

Asp Thr Gly Thr Arg Thr Arg His Ile Thr Gln Gly Thr Glu Ser Phe
770 775 780

Pro Ile Ser Ser Met Glu Ser Glu Gln Lys Arg Ile Gly Gly Ala Gly
785 790 795 800

Ser Leu

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

ATGAAGAAGA AYAAYAAGGG YGAG 24

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

TARTCRTTCT TCTTRTCCTC RTG 23

Claims

1. An isolated nucleotide sequence comprising DNA encoding a protein, wherein said protein is selected from the group consisting of *CAP85*, *CAP160*, and fragments thereof, wherein said fragments enhance cold tolerance or drought resistance of a cell.

2. The nucleotide sequence, according to claim 1, wherein said encoded protein is *CAP85* or a fragment of said *CAP85* protein.

3. The nucleotide sequence, according to claim 2, wherein said encoded protein comprises the amino acid sequence shown in SEQ ID NO. 2, or a portion of said amino acid sequence.

4. The nucleotide sequence, according to claim 2, wherein said encoded protein comprises the DNA sequence shown in SEQ ID NO. 1, or a portion of said DNA sequence.

5. The nucleotide sequence, according to claim 1, wherein said encoded protein is *CAP160*, or a fragment of said *CAP160*.

6. The nucleotide sequence, according to claim 5, wherein said encoded protein comprises the amino acid sequence shown in SEQ ID NO. 4, or a portion of said amino acid sequence.

7. The nucleotide sequence, according to claim 5, wherein said encoded protein comprises the DNA sequence shown in SEQ ID NO. 3, or a portion of said DNA sequence.

8. A process for enhancing cold tolerance or drought resistance of a cell, wherein said process comprises transforming said cell with a polynucleotide sequence comprising DNA which codes for a cold acclimation protein selected from the group consisting of *CAP85*, *CAP160*, and fragments thereof, wherein said fragments enhance cold tolerance or drought resistance of said cell.

9. The process, according to claim 8, wherein said cell is a plant cell.

10. The process, according to claim 9, wherein said plant cell is a cell of a plant of the family Solanaceae.

11. The process, according to claim 9, wherein said plant cell is a citrus plant cell.

12. The process, according to claim 8, wherein said cell is a bacterium.

13. The process, according to claim 8, wherein said microorganism is a yeast cell.

14. A transformed cell, wherein said cell is transformed according to the process of claim 8.

15. The transformed cell, according to claim 14, wherein said cell is a plant cell.

16. The transformed cell, according to claim 15, wherein said plant cell is a citrus plant cell.

17. A process for protecting a plant cell from freeze damage or desiccation damage, wherein said process comprises applying to the situs or growth medium of the plant cell a protein selected from the group consisting of *CAP85*, *CAP160*, and fragments of *CAP85* or *CAP160*, wherein said fragments protect the plant cell from freeze damage or desiccation damage.

18. The process, according to claim 17, wherein said protein is *CAP85*.

19. The process, according to claim 17, wherein said protein is *CAP160*.

20. A process for protecting a microorganism against freeze damage or desiccation damage, wherein said process comprises applying to the situs or culture medium of said microorganism a cold acclimation protein, wherein said protein is selected from the group consisting of *CAP85*, *CAP160*, and a fragment of *CAP85* or *CAP160*, wherein said fragment protects a microorganism from freeze damage or desiccation damage.

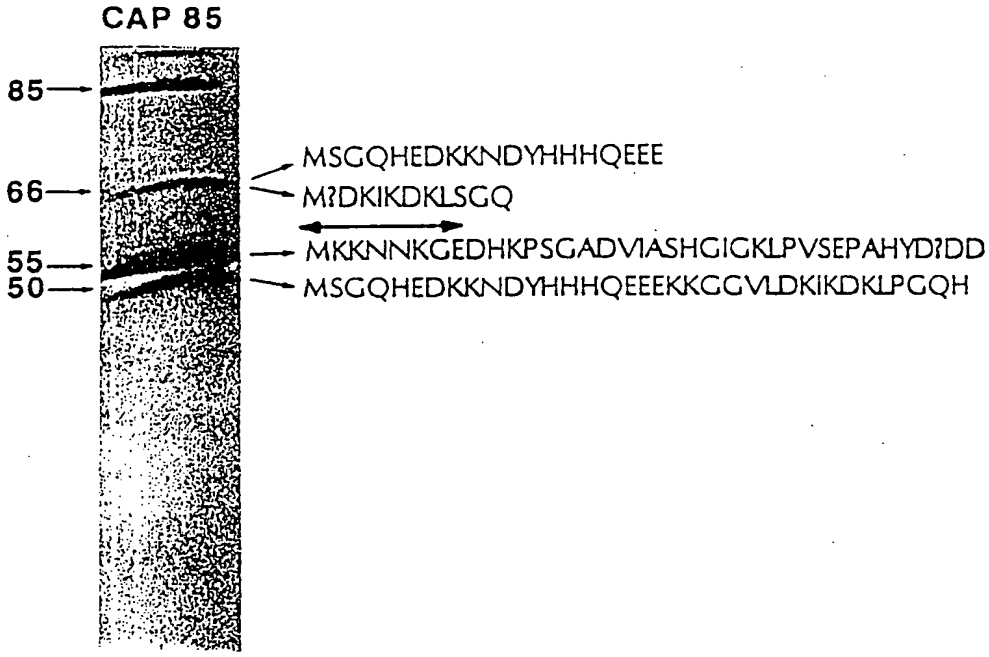

CAP 85
85
66
55
50
MSGQHEDKKNDYHHHQEEE
M?DKIKDKLSGQ
MKKNNKGEDHKPSGADVIASHGIGKLPVSEPAHYD?DD
MSGQHEDKKNDYHHHQEEEKKGGVLDKIKDKLPGQH

Fig. 1

```
ATGAAGAAGAATAACAAGGGTGAGGATCACAAGCCATCCGAGGCCGATGT    50
M  K  K  N  N  K  G  E  D  H  K  P  S  E  A  D  V

GATTGCCTCCGGCGGTATCGGAAAGTTGCCCGTCTCCGAACCTGCTCATT   100
 I  A  S  G  G  I  G  K  L  P  V  S  E  P  A  H  Y

ATGACCATGATGACAAGGAACATGTTGGACTCCTTGAGAAAAAACATATT   150
  D  H  D  D  K  E  H  V  G  L  L  E  K  K  H  I

GGACTTGTTGAGCAATTCCATCGTTCTGATCACGCTTCCGACGAAAGACA   200
G  L  V  E  Q  F  H  R  S  D  H  A  S  D  E  R  H

TCATGATGAAGAGCAAAACAAAGGTGGTGTCTTCGGAAAAATCAAGGAGA   250
 H  D  E  E  Q  N  K  G  G  V  F  G  K  I  K  E  K

AGCTCCCCGGTCAGCATGATTCGGATACTACCACACATACACAACAATTA   300
  L  P  G  Q  H  D  S  D  T  T  T  H  T  Q  Q  L

TACCCTGCTTCTGATCATAACTACAACACCCACCATGTCCACCAAGACGA   350
Y  P  A  S  D  H  N  Y  N  T  H  H  V  H  Q  D  D

TGAAAAGAAGGACAACATCCTTGACAAAATCAAGGATAAGCTTCCCGGAA   400
 E  K  K  D  N  I  L  D  K  I  K  D  K  L  P  G  K

AACACGAAGATAAGAAGCAAGACTATCACCAGCACCAAGAGGAGGAAAAG   450
  H  E  D  K  K  Q  D  Y  H  Q  H  Q  E  E  E  K

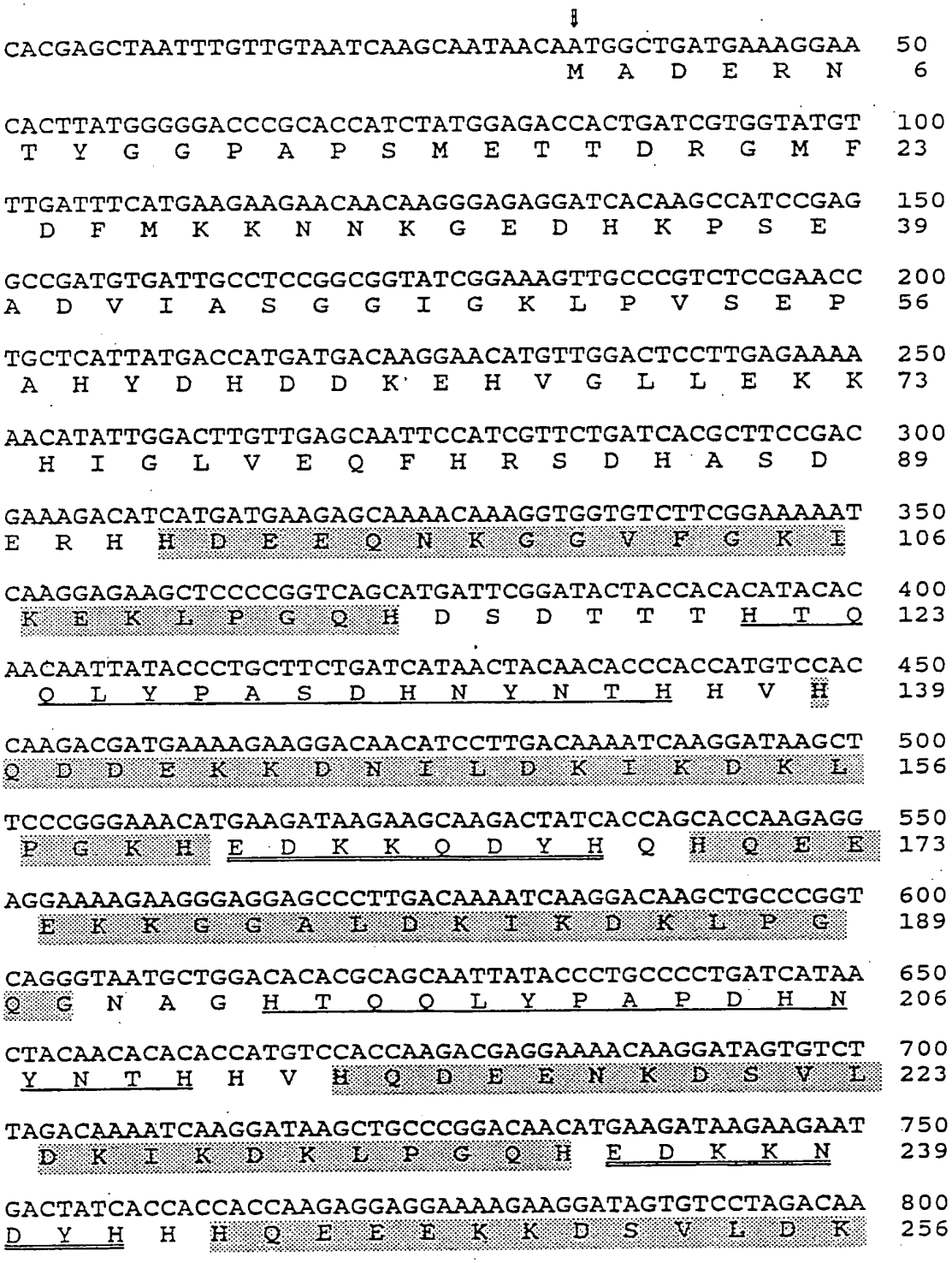
AAGGGAGGAGCCCTTGACAAAATCAAGGACAAGCTGCCCGGTCAGGGTAA   500
K  G  G  A  L  D  K  I  K  D  K  L  P  G  Q  G  N

TGCTGGACACACGCAGCAATTATACCCTGCCCCTGATCATAACTACAACA   550
 A  G  H  T  Q  Q  L  Y  P  A  P  D  H  N  Y  N  T

CACACCATGTCCACCAAGACGAGGAAAACAAGGATAGTGTCTTAGACAAA   600
  H  H  V  H  Q  D  E  E  N  K  D  S  V  L  D  K

ATCAAGGATAAGCTGCCCGGACTACATGAGGACAAGAAGAACGACTAT     648
I  K  D  K  L  P  G  L  H  E  D  K  K  N  D  Y
```

Fig. 2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M A D E R N 6
CACTTATGGGGGACCCGCACCATCTATGGAGACCACTGATCGTGGTATGT 100
T Y G G P A P S M E T T D R G M F 23
TTGATTTCATGAAGAAGAACAACAAGGGAGAGGATCACAAGCCATCCGAG 150
D F M K K N N K G E D H K P S E 39
GCCGATGTGATTGCCTCCGGCGGTATCGGAAAGTTGCCCGTCTCCGAACC 200
A D V I A S G G I G K L P V S E P 56
TGCTCATTATGACCATGATGACAAGGAACATGTTGGACTCCTTGAGAAAA 250
A H Y D H D D K E H V G L L E K K 73
AACATATTGGACTTGTTGAGCAATTCCATCGTTCTGATCACGCTTCCGAC 300
H I G L V E Q F H R S D H A S D 89
GAAAGACATCATGATGAAGAGCAAAACAAAGGTGGTGTCTTCGGAAAAAT 350
E R H H D E E Q N K G G V F G K E 106
CAAGGAGAAGCTCCCCGGTCAGCATGATTCGGATACTACCACACATACAC 400
K E K L P G Q H D S D T T T H T Q 123
AACAATTATACCCTGCTTCTGATCATAACTACAACACCCACCATGTCCAC 450
Q L Y P A S D H N Y N T H H V H 139
CAAGACGATGAAAAGAAGGACAACATCCTTGACAAAATCAAGGATAAGCT 500
Q D D E K K D N I L D K I K D K L 156
TCCCGGGAAACATGAAGATAAGAAGCAAGACTATCACCAGCACCAAGAGG 550
P G K H E D K K Q D Y H Q H Q E E 173
AGGAAAAGAAGGGAGGAGCCCTTGACAAAATCAAGGACAAGCTGCCCGGT 600
E K K G G A L D K I K D K L P G 189
CAGGGTAATGCTGGACACACGCAGCAATTATACCCTGCCCCTGATCATAA 650
Q G N A G H T Q Q L Y P A P D H N 206
CTACAACACACACCATGTCCACCAAGACGAGGAAAACAAGGATAGTGTCT 700
Y N T H H V H Q D E E N K D S V L 223
TAGACAAAATCAAGGATAAGCTGCCCGGACAACATGAAGATAAGAAGAAT 750
D K I K D K L P G Q H E D K K N 239
GACTATCACCACCACCAAGAGGAGGAAAAGAAGGATAGTGTCCTAGACAA 800
D Y H H H Q E E E K K D S V L D K 256

Fig. 3A

```
AATCAAGGATAAGATGTCCGGGCAGCATGAAGATAAGAAGAATGACTATC   850
 I  K  D  K  M  S  G  Q  H  E  D  K  K  N  D  Y  H   273

ACCACCACCAAGAGGAGGAAAAGAAGGGAGGAGTCCTTGACAAAATCAAG   900
   H  H  Q  E  E  E  K  K  G  G  V  L  D  K  I  K    289

GACAAGTTGCCTGGTCAACATGATGCAGACACTGCCAGACACACGCAGCA   950
D  K  L  P  G  Q  H  D  A  D  T  A  R  H  T  Q  Q   306

ACTATACCCTGCTGCTGATCATAACTACAACACACCATGTCCACCAAG    1000
 L  Y  P  A  A  D  H  N  Y  N  T  H  H  V  H  Q  D  323

ATGAGGAAAACAAGGATAGCGTCCTTGACAAAATCAAGGACAAACTACCC  1050
   E  E  N  K  D  S  V  L  D  K  I  K  D  K  L  P    339

GGACAACATGATGATAAGGCTGCATACTCGCAACATGACCACCACAAGCA  1100
G  Q  H  D  D  K  A  A  Y  S  Q  H  D  H  H  K  H   356

CCACCAAGAGGAGGAAAACAAGGGTGGAGTCCTCGACAAAATCAAGGACA  1150
 H  Q  E  E  E  N  K  G  G  V  L  D  K  I  K  D  K  373

AACTGCCTGGTGTCTACATGGTGGTCAAACATGATGGTGATATTGTCGAA  1200
   L  P  G  V  Y  M  V  V  K  H  D  G  D  I  V  E    389

CACACGCAACAATTATACCCTGCTCCTGATCATAACTACAACACTCACTA  1250
H  T  Q  Q  L  Y  P  A  P  D  H  N  Y  N  T  H  Y   406

TGTCCATGAAGACGAGAAAAAGAAGGATAGTGTCCTAGACAAAATCAAGG  1300
 V  H  E  D  E  K  K  K  D  S  V  L  D  K  I  K  D  423

ACAAGTTACCCGGACAACATGAGGAAAAGGCAGCAGCATACTCTGAGCCA  1350
   K  L  P  G  Q  H  E  E  K  A  A  A  Y  S  E  P    439

TCATATGATTCACACCCTACACCTGCAAAGCATCATGATTATTTCCCCCA  1400
S  Y  D  S  H  P  T  P  A  K  H  H  D  Y  F  P  Q   456

AGAGGAGGAAAAGAAAGGTGGTGTCATGGACAAAATTAAGGACAAGCTTT  1450
 E  E  E  K  K  G  G  V  M  D  K  I  K  D  K  L  S  473

CCGGCCAACATAAAGATAAGGCCGACGAGCATGAGTTGGTTGCTCCGTTG  1500
  G  Q  H  K  D  K  A  D  E  H  E  L  V  A  P  L    489

GTGACAGTCGAACCACATTCTGAGGGTGATAAGGAAAAGAAGGGGTTCTT  1550
V  T  V  E  P  H  S  E  G  D  K  E  K  K  G  F  L   506

GGAGAAGATTAAGGACAAAATCCCCGGCCTCCACTCCAAGAATGATGCTG  1600
 E  K  I  K  D  K  I  P  G  L  H  S  K  N  D  A  E  523

AAGAGAAGAAGACCCATGAGGAGAAAAAAGAGGGATACTAAACTTAACTA  1650
  E  K  K  T  H  E  E  K  K  E  G  Y  .              535
```

Fig. 3B

I=	H	D	E	E	Q	N	K	G	G	V	F	G	K	I	K	E	K	L	P	G	Q	H
II=	H	Q	D	D	E	K	K	D	N	I	L	D	K	I	K	D	K	L	P	G	K	H
III=	H	Q	E	E	E	K	K	G	G	A	L	D	K	I	K	D	K	L	P	G	Q	G
IV=	H	Q	D	E	E	N	K	D	S	V	L	D	K	I	K	D	K	L	P	G	Q	H
V=	H	Q	E	E	E	K	K	D	S	V	L	D	K	I	K	D	K	M	S	G	Q	H
VI=	H	Q	E	E	E	K	K	G	G	V	L	D	K	I	K	D	K	L	P	G	Q	H
VII=	H	Q	E	E	E	K	K	G	G	V	L	D	K	I	K	D	K	L	P	G	Q	H
VIII=	H	Q	E	E	E	N	K	G	G	V	L	D	K	I	K	D	K	L	P	G	V	Y
IX=	H	E	D	E	K	K	K	D	S	V	L	D	K	I	K	D	K	L	P	G	Q	H
X=	P	Q	E	E	E	K	K	G	G	V	M	D	K	I	K	D	K	L	S	G	Q	H
XI=	S	E	G	D	K	E	K	K	G	F	L	E	K	I	K	D	K	I	P	G	L	H
CONSENSUS	H	Q	E	E	E	K	K	G	G	V	L	D	K	I	K	D	K	L	P	G	Q	H
RESIDUE	+	p	-	-	-	+	+	g	g	n	n	-	+	n	+	-	+	n	p	g	p	+

Fig. 4

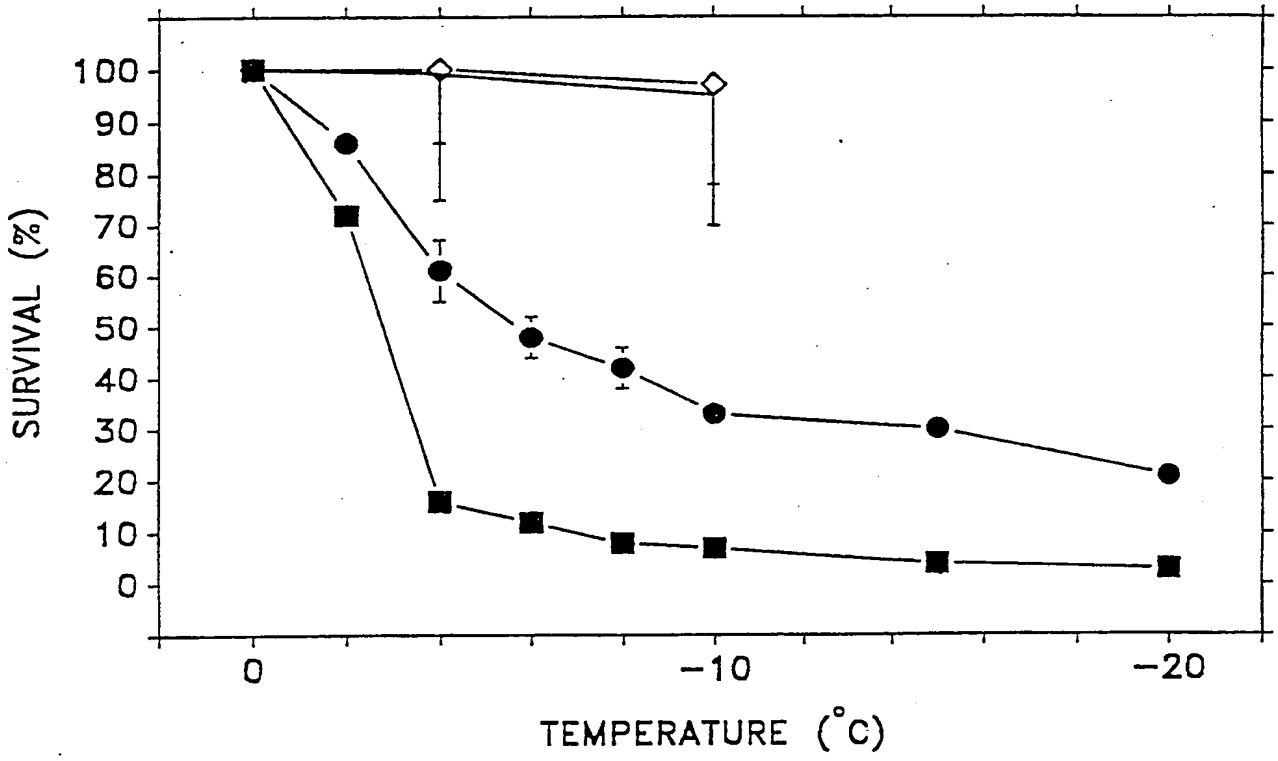

Fig. 5

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 94/00581

A. CLASSIFICATION OF SUBJECT MATTER
IPC 5 C12N15/29 C12N15/82 C12N5/10 C12N1/21 A01N3/00
C12N1/04

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 5 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
O,X	J. CELL. BIOCHEM. SUPPL. vol. 13D , 1989 page 319 HAJELA, R.K., ET AL. 'Cloning of cold regulated genes from Arabidopsis thaliana' see abstract M417	1,5
X	PLANTA vol. 188 , 1992 pages 265 - 270 GUY, C., ET AL. 'Hydration-state-responsive proteins link cold and drought stress in spinach' see page 266, right column	1,2
Y		1-20

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
20 June 1994	01. 07. 94

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Maddox, A

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	PLANT PHYSIOL. BIOCHEM. vol. 27 , 1989 pages 777 - 784 GUY, C.L., ET AL. 'Preliminary characterization of high moleecular mass proteins associated with cold acclimation in spinach' see page 783, right column	1-7
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A	PLANT MOLECULAR BIOLOGY. vol. 18 , 1992 , DORDRECHT, THE NETHERLANDS. pages 13 - 21 GILMOUR, S.J., ET AL. 'cDNA analysis and expression of two cold-regulated genes of Arabidopsis thaliana' see the whole document	1-20

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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A	PLANT MOLECULAR BIOLOGY. vol. 20 , 1992 , DORDRECHT, THE NETHERLANDS. pages 555 - 558 ITURRIAGA, G., ET AL. 'Expression of dessication-related proteins from the resurrection plant Craterostigma plantagineum in transgenic tobacco' see the whole document	8-16
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INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 94/00581

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
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		JP-T-	4505329	17-09-92
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		EP-A-	0356061	28-02-90
		EP-A-	0382834	22-08-90
		JP-T-	3502887	04-07-91
WO-A-8909267	05-10-89	AU-B-	626012	23-07-92
		AU-A-	3356389	16-10-89
		EP-A-	0335528	15-11-89
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